University of Cape Town

Institute of Infectious Diseases and Molecular Medicine

Investigating cross-clade immune responses in HIV-1 subtype C-infected individuals from South Africa

By

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A dissertation submitted in fulfilment of the requirements for the degree of MSc (Med) in the Department of Clinical Laboratory Sciences, Division of Medical Virology, University of Cape Town

August 2007

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ACKNOWLEDGEMENTS

First and foremost, I offer my sincere gratitude to my supervisor Dr Wendy Burgers and co-supervisor Prof Carolyn Williamson who supported me throughout my thesis with their patience and knowledge whilst allowing me the room to think on my own. I attribute the level of my Masters Degree to their encouragement and effort.

I would like to acknowledge Prof Clive Gray, our research collaborator who without fail kindly provided immunology reagents, advice and insight into the project reported in this thesis. Many thanks go to Helba Bredell for her technical help and to the whole HIV Diversity Group for their friendly environment.

I would like to express my deepest appreciation to Kathi, Richard, Phillipa and David and family who have the substance of good parents for their invaluable support.

My sincere appreciation goes to South African AIDS Vaccine Initiative (SAAVI), Poliomyelitis Research Foundation (PRF) and the Beit Student Support Fund for their financial support and International AIDS Vaccine Initiative (IAVI) for funding the project.

DECLARATION

I, **Lycias Zembe**, hereby declare that the work on which this thesis is based is my own work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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LIST OF ABBREVIATIONS

ABI	- Applied Biosystems
Bp	- Base pair
cDNA	- Complementary deoxyribonucleic acid
CD4	- Cluster of differentiation 4
CD8	- Cluster of differentiation 8
CEF	- Cytomegalovirus, Epstein-Bar virus and Influenza virus
CI	- Confidence interval
CPT	- cell preparatory tube
CTL	- Cytotoxic T-lymphocyte
C_{CH}	- Chinese subtype C sequence
$C_{S.A}$	- South African subtype C Du422 sequence
dH_2O	- distilled water
DMSO	- Dimethylsulphoxide
ddNTP	- Dideoxynucleotide triphosphate
DECP	- Diethyl pyrocarbonate
DNA	- Deoxyribonucleic acid
DNases	- Deoxyribonucleases
dNTP	- Deoxynucleotide triphosphate
EDTA	- ethylenediaminetetraacetic acid
ELISpot	- Enzyme Linked ImmunoSpot assay
Env	- Envelope
EtBr	- Ethidium bromide
FCS	- Foetal Calf Serum
Gag	- Group specific antigen
HIV-1	- Human immunodeficiency virus type 1
HLA	- Human leukocyte antigen
HRP	- Horseradish Peroxidase
IAVI	- International AIDS Vaccine Initiative
IFN-γ	- Interferon gamma
IIDMM	- Institute of Infectious Diseases and Molecular Medicine
IN	- Integrase
kD	- KiloDalton
LTR	- Long terminal repeat

MEGA	- Molecular Evolutionary Genetics Analysis
MHC	- Major Histocompatibility Complex
mM	- Milimolar
ml	- Millilitre
NICD	- National Institute for Communicable Diseases
NJ	- Neighbor Joining
PAUP	- Phylogenetic Analysis Using Parsimony
PBMC	- Peripheral Blood mononuclear cells
PBS	- Phosphate Buffer Saline
PCR	- Polymerase chain reaction
PHA	- Phytohaemagglutinin
Pmol	- Picomole
Pol	- Polymerase
QC	- Quality control
RCF-*g*	- Relative Centrifugal Force *g*
Rev	- Regulator of Virion
RNA	- ribonucleic acid
RNases	- Ribonucleases
RPM	- Revolution per minute
RPMI	- Roswell park Memorial Institute media
RT	- Room temperature (15^0C-25^0C)
RT	- Reverse Transcriptase
R10	- RPMI media with 10% FCS
R20	- RPMI media with 20% FCS
SAAVI	- South African AIDS Vaccine Initiative
SFU/10^6 PBMCs	- Spot forming units per million PBMCs
TBE	- Tris Boric acid EDTA buffer
UV	- Ultraviolet
Vif	- Viral infectivity factor
Vpr	-Viral protein R
Vpu	- Viral protein u
µg	- Microgram
µl	- Microlitre
^{0}C	- Degrees Celsius

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ABSTRACT

The increasing diversity of HIV-1 in different geographic regions presents a challenge for the development of an HIV/AIDS vaccine. Even if proven effective, the ability of a vaccine to have cross-subtype and intra-subtype protection remains an important question. The aim of the study was to investigate cross- and intra-clade T cell immune responses in HIV-1 subtype C-infected individuals. The objectives were to genetically characterise full length *gag* gene sequences from individuals chronically infected with HIV-1 and then assess the degree of cross-reactive immune responses in an ELISpot assay using peptide reagents based on vaccine candidates. Forty HIV-infected antiretroviral therapy naïve individuals were recruited into the study. The HIV-1 *gag* region was amplified and directly sequenced. Phylogenetic analysis was performed to determine the subtype of the infecting strain and genetic distances calculated between isolate sequences and peptide reagent sequences used in immunological assays. Cross-clade T cell immune responses were then examined in the peripheral blood mononuclear cells of 39 study subjects in an Enzyme Linked Immuno Spot (ELISpot assay). An ELISpot assay making use of a pool and matrix approach allowed for detection of responses to peptides spanning the Gag protein from two subtype Cs, subtypes B, A and D, based on vaccine candidates. The reactive peptides were confirmed in a second ELISpot assay in two individuals. All viruses were subtype C viruses and their sequences were not more closely related to each other when compared to other subtype C viruses. The p24 region was the most highly conserved among study sequences as expected. HIV Gag-specific T cell responses were detected in 97.4% (38/39) of the study individuals. All of the individuals who had detectable HIV-specific T cell responses recognized Gag peptides based on $C_{S.A}$ and C_{CH} sequences. Interestingly, there was no significant difference in the magnitude of T cell response to the two subtype C sequences. The total magnitude of the HIV-specific T cell responses to the two subtype C sequences was significantly higher than that of subtypes B, A and D. 78.9% of the individuals had anti-HIV T cell responses to all 5 HIV-1 subtypes. 7.9%, 5.3% and 7.9% of the individuals responded to 4, 3 and 2 subtypes, respectively. All individuals who had T cell responses recognized at least two HIV peptide variants. The cross-clade responses were substantial for the p24 region of Gag protein. In the individuals where reactive peptides were confirmed, HIV-specific T cell responses were directed against peptides with lower intra-clade entropy and simultaneously high inter-clade homology. These data demonstrate that

HIV-1 subtype C-infected individuals can mount vigorous intra- and cross-clade immune responses against Gag from subtypes A, B, C and D. Therefore, these results suggest that vaccines based on these two subtype C sequences may work equally well. However, vaccines based on other subtypes might be less effective than clade-matched vaccines. The data might be useful in making decisions about testing candidate vaccines in regions where different HIV-1 subtypes than those in the vaccine are circulating.

CHAPTER 1

Literature review

1.1. General

The number of people infected with HIV worldwide is estimated to be 42 million, with more than 90% living in developing countries, 10% of whom are in South Africa (UNAIDS: AIDS epidemic update 2007). Although antiretroviral therapy has become increasingly available, access in Africa remains limited and an effective vaccine remains our greatest hope for controlling this epidemic. There is, therefore, a desperate global need for a prophylactic HIV vaccine.

There are a number of major scientific challenges in developing such a vaccine, of which genetic diversity is one of them. HIV-1 is a highly diverse virus with at least nine subtypes, as well as thirty-four intersubtype circulating recombinant forms which have been described to date (http://hiv-web.lanl.gov), of which three, namely CRF01_AE, CRF07_BC and CRF08_BC, have played a critical role in the Asian HIV-1 epidemic. The emergence of unique recombinant forms adds to the enormous genetic diversity of HIV-1, which has implications for HIV vaccine development (Casado *et al.*, 2005; Robertson *et al.*, 1995; 2000). Continued monitoring of the genetic diversity of HIV and the global distribution of the different subtypes is necessary to inform vaccine development strategies.

It is not known whether the T cell vaccine concept will be successful at inducing strong HIV-specific T cell immune responses that can control HIV replication. This is illustrated by the recent failure of the Merck Ad5 vaccine in phase IIb vaccine trials, in which vaccinated individuals were more susceptible to HIV infection than unvaccinated individuals (Cohen, 2007a; 2007b). If the T cell vaccine concept could be successful at controlling HIV replication, it is important to know whether the immune responses generated could be cross-reactive among the different HIV subtypes. In other words, vaccine researchers need to know whether clade (subtype)-matched vaccines are necessary, which is of great importance in deciding whether vaccines based on certain subtypes can be tested in populations where a different subtype is circulating.

Although the immune correlates of protection in HIV infection are not fully understood, numerous studies suggest that the induction of both HIV-specific cellular and humoral immune responses is important for an effective vaccine against HIV. Furthermore, an understanding of cellular immune responses to different HIV subtypes, the viral regions

that are preferentially recognized and host genetic factors involved in those responses will be of importance in vaccine development approaches. The aim of the study reported in this thesis was to measure cross-reactive immune responses to different HIV subtypes in individuals infected with a single subtype (termed 'cross-clade' responses), in order to predict whether a future vaccine based on one subtype may be cross-reactive, and therefore potentially effective, in a population where a different subtype is circulating.

1.2. Structure and genomic organization of HIV-1

The Human Immunodeficiency virus (HIV) belongs to the family of retroviruses, subgroup lentiviruses (Sharp *et al.*, 2000). Other lentiviruses include Simian Immunodeficiency Virus (SIV), Feline immunodeficiency Virus (FIV), Visna and Caprine Arthritis-Encephalitis Virus (CAEV), which cause diseases in monkeys, cats, sheep and goats (Nishimura *et al.*, 1999; Pasick, 1998). Infections with lentiviruses typically show a chronic course of disease, a long period of clinical latency, persistent viral replication and involvement of the central nervous system. HIV, which causes AIDS, was first isolated in 1983 (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1988).

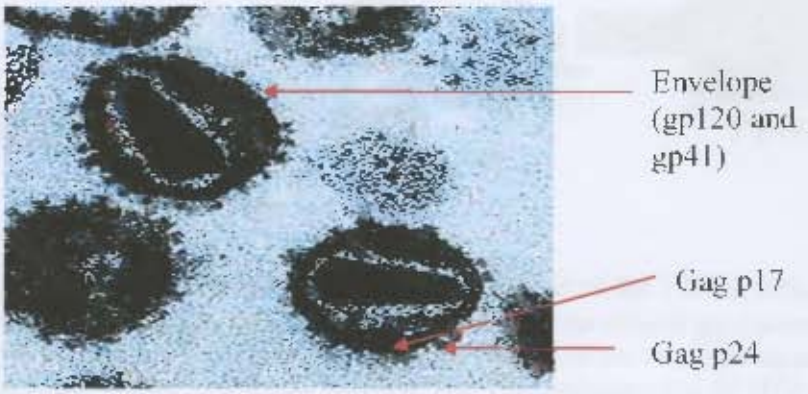


Figure 1.1 HIV-1 electron micrograph. The Envelope proteins gp120 and gp41 together make up the spikes that project from the HIV particle, while Gag p17 forms the matrix and Gag p24 the core. The size of an HIV particle is around 0.1 microns. Adapted from Gallo and Montagnier, 1988)

Outside of a human cell, HIV exists as roughly spherical viral particles or virion (Gelderblom, 1991). The HIV envelope protein protrudes from the surface of virions giving the appearance of spikes as shown by the photograph in Figure 1.1 and diagrammatically in Figure 1.2 (a). HIV has a diploid RNA genome approximately 9.7 kb in length and consists of nine genes that code for at least fifteen different proteins (Figure

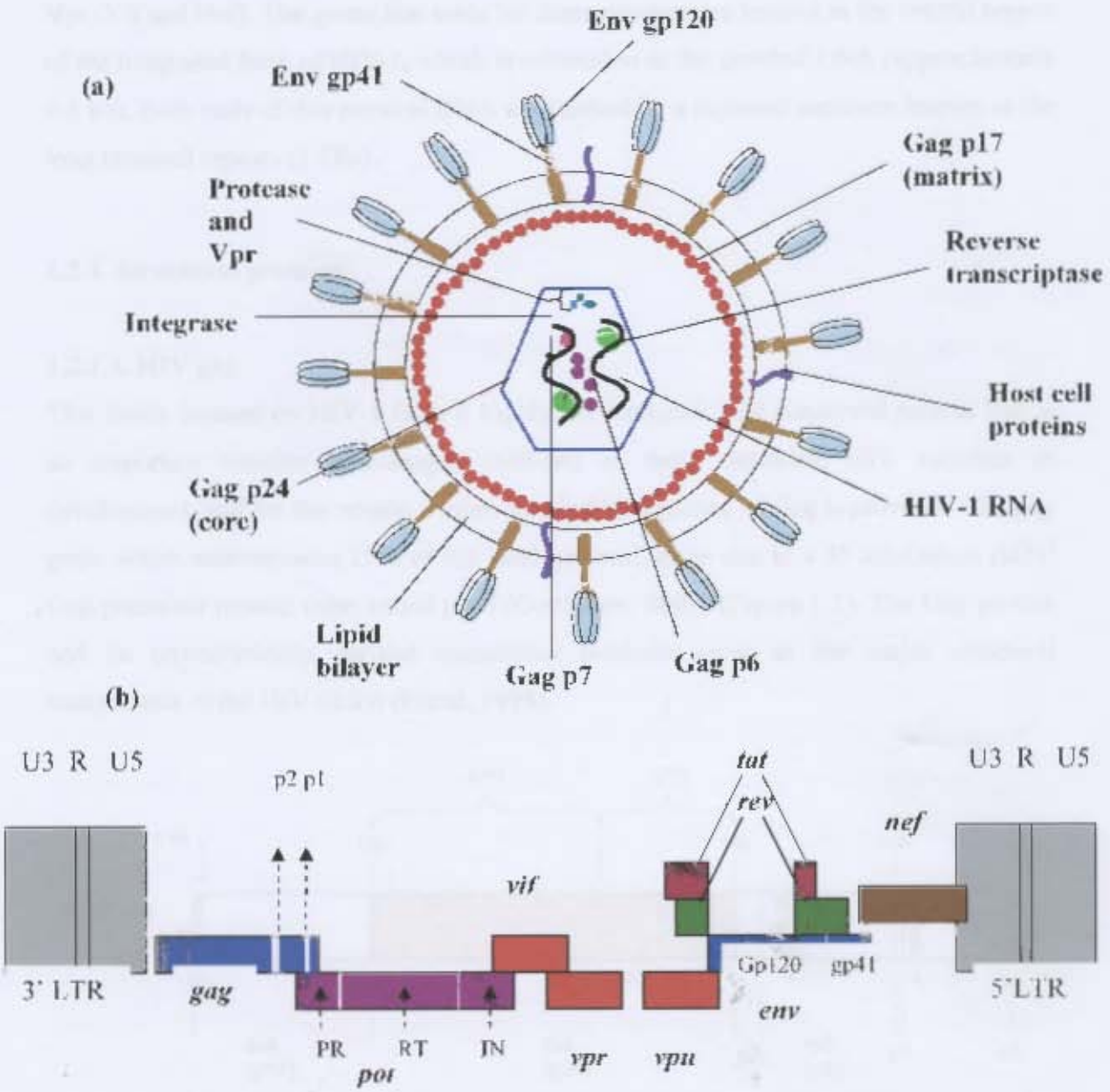


Figure 1.2 (a) Diagrammatic representation of the morphologic structure of the HIV-1 virion particle. In a mature virion, MA forms a layer beneath the host-derived lipid bilayer. The trimeric glycoprotein spikes composed of gp120 anchored to the lipid bilayer membrane by gp41 are exposed on the virion surface and are involved in the attachment of the virion to CD4 cells during infection. (b) The organization of HIV-1 genome. The HIV-1 proviral genome (9.7 kb) is composed of nine genes (italicized and bold) that are flanked by two long terminal repeats (LTRs). The nine open reading frames encode at least 15 proteins. The *gag* gene is translated into the structural Gag precursor (Pr 55 ^{Gag}) which is cleaved into matrix (p17), capsid (p24), (which coats the viral single stranded RNA genome), p7 (nucleocapsid which binds to the HIV packaging signal on viral RNA and is sufficient for incorporation of RNA into virions), and p6 (p2 and p1 are spacer peptides). The *pol* gene, translated by (-1) ribosomal frameshift as a Gag-Pol precursor polypeptide that yields the three enzymes reverse transcriptase (RT), integrase (IN) and protease (PR) upon cleavage. The *env* gene encodes anchor structural precursor (env, gp160), which is cleaved into the surface (gp120) and transmembrane (gp41) glycoprotein. *Gag-Pol* mRNAs are spliced to encode the regulatory (rev, tat, nef) and accessory (vpr, vpu, vif) proteins. Diagram drawn based on information from http://hiv-web.lanl.gov.

1.2 b). These proteins can be divided into three main classes; the major structural proteins (Gag, Pol and Env), the regulatory proteins (Tat and Rev), the accessory proteins (Vpu,

Vpr, Vif and Nef). The genes that code for these proteins are located in the central region of the integrated form of HIV-1, which is referred to as the proviral DNA (approximately 9.8 kb). Both ends of this proviral DNA are flanked by a repeated sequence known as the long terminal repeats (LTRs).

1.2.1. Structural proteins

1.2.1.1. HIV gag

This thesis focuses on HIV-1 Gag, a highly immunogenic and conserved protein that is an important vaccine immunogen included in most candidate HIV vaccines in development, and for this reason a more detailed background of Gag is provided. The *gag* gene, which encompasses 15% of the viral genome, gives rise to a 55 kiloDalton (kDa) Gag precursor protein (also called p55) (Gottlinger, 2001) (Figure 1.3). The Gag protein and its proteolytically derived maturation products serve as the major structural components of the HIV virion (Freed, 1998).

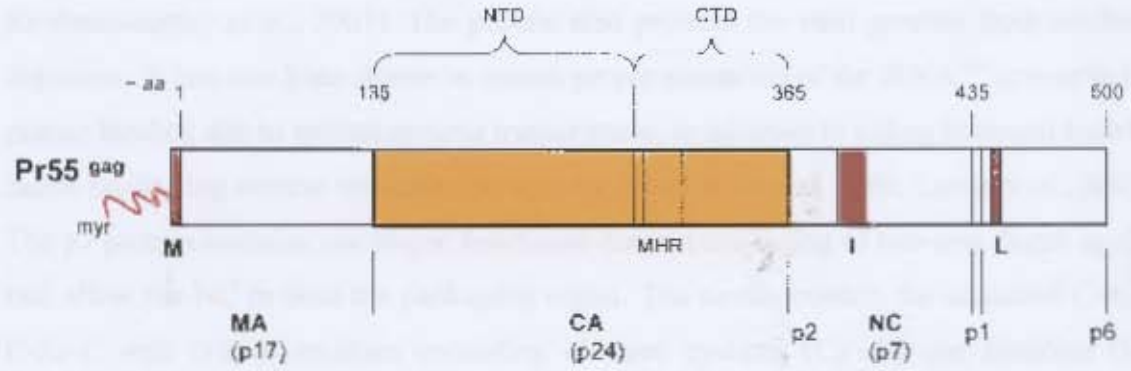


Figure 1.3 A schematic representation of Pr55 ^{gag} and its sub-domains. The positions of the different Gag domains according to HXB2 (a commonly used reference strain based on HIV-1 subtype B) Gag protein sequence are indicated. Abbreviations used: NTD (Amino-terminal domain), CTD (Carboxy (C)-terminal domain), aa (amino acid), MHR (major homology region), M (membrane binding-domain), I (Interaction-domain) and L (Late-domain). The p24 has been shown to be more conserved than other Gag regions.

p17 (matrix)

The matrix protein is derived from the N-terminal, myristylated end of p55 and forms the scaffold between the cores and the outer envelope. Myristylation is the co-translational addition of a 14-carbon fatty acid (myristate) that is associated with targeting of proteins to membranes. The p17 region also contains a nuclear localization domain which facilitates the nuclear transport of the viral genome. This allows HIV to infect non-dividing cells, an unusual property of a retrovirus. The M-domain maps to the first 32

amino acids of Gag and includes the terminal myristate (Zhou *et al.*, 1994). Deletions in the M-domain abolish membrane binding and budding.

p24 (capsid)

The p24 protein is a highly conserved region of the HIV Gag protein that forms the conical core of viral particles (Gottlinger, 2001). Cyclophilin A, a cellular protein, has been demonstrated to interact with p24 and can be incorporated into HIV particles. The p24 region has been shown to be essential for the proper folding of the virion proteins (Yoo *et al.*, 1997). It is the p2 domain next to the capsid that has been demonstrated to confer sensitivity of the capsid protein to Cyclophilin A binding (Dorfman & Gottlinger, 1996).

Nucleocapsid, NC (p7)

The NC region of Gag is an important gene for the transmission and pathogenesis of the virus. It is responsible for specifically recognizing the packaging signal of HIV, packaging two copies of the viral genome into progeny virions (Heath *et al.*, 2003; Krishnamoorthy *et al.*, 2003). The protein also protects the viral genome from nuclease digestion. It has also been shown to ensure proper annealing of the $tRNA^{Lys}$ primer to the primer binding site to initiate reverse transcriptase, in addition to aiding in strand transfer hence facilitating reverse transcriptase enzyme function (Freed, 1998; Levin *et al.*, 2005). The p7 protein contains one major functional domain consisting of two zinc finger motifs that allow the NC to bind the packaging signal. The motifs contain the sequence $C\text{-}X_2 - C\text{-}X_4\text{-}C$ with critical residues consisting of three cystines (C) and one histidine (H). Mutation of these amino acid residues to non-zinc binding residues results in virions defective in RNA packaging (Freed, 1998; McGrath *et al.*, 2003). The antiviral effects of APOBEC3G (a cytidine deaminase that introduces G→A hypermutations in newly synthesized viral DNA) have been shown to be through interaction with the p7 region of HIV Gag protein (Alce & Popik, 2004). HIV vif, an accessory protein has been shown to interact with APOBEC3G thereby overcoming the antiviral effects of the enzyme. Furthermore, mutations in the p7 protein may lead to lack of antiviral effects by APOBEC3G. Therefore, any alterations to the gene may affect transmission and pathogenesis of the virus.

p6

A proline-rich peptide of approximately 6 kDa derived from the C terminus of the p55 Gag precursor protein is called p6. The p6 region mediates the interaction between p55 Gag and the accessory protein Vpr, leading to Vpr incorporation into the assembling virions (Zhu *et al.*, 2004). The protein also contains the late domain which is required for the efficient release of budding virions from an infected cell. Mutations of this protein have been shown to prevent the release of budded virus particles from the cell surface (Freed, 2002).

1.2.1.2. Pol

During the replication cycle of HIV-1, the single stranded RNA (ssRNA) genome is reverse transcribed by the HIV enzyme reverse transcriptase (RT), coded for by the *pol* gene (Kuiken *et al.*, 2000). *Pol* also encodes for the enzymes protease, integrase and RNAse H, all of which are essential for replication (Coffin, 1990). Protease processes proteins made from HIV's genome so that they can become part of newly fully-functional HIV particles (Short *et al.*, 2000). RNAse-H breaks down the retroviral genome after being copied into DNA by reverse transcriptase following infection of a cell. The fourth Pol protein, Integrase, catalyzes integration of the viral provirus into the host genome (Craigie, 2001; Wang *et al.*, 2001).

1.2.1.3. Env

Env consists of two proteins, gp120 and gp41. Gp120 is located on the outside of the virus while each gp41 molecule is anchored to a gp120 through the membrane. The Envelope protein exists as a trimer and is responsible for recognition of cellular receptors and viral entry into cells.

1.2.2. Regulatory proteins

1.2.2.1. Tat

Tat (transactivator) is a 101 kDa regulatory protein that accelerates the production of HIV viral particles. It acts as a transcriptional regulator of viral gene expression by binding to the transactivating responsive sequence (TAR) RNA element. This initiates viral transcription and/ or elongation from long terminal repeat promoter (Roy *et al.*, 1990). Tat also up-regulates the expression of all viral genes and promotes the elongation phase of HIV-1 transcription, allowing full-length transcripts to be produced (Feinberg *et al.*, 1991). This effect of Tat is different in different HIV-1 subtypes suggesting that the

differential critical role of Tat for viral infectivity and pathogenesis in different HIV-1 subtypes may differentially modulate the pathogenic properties of the different viral subtypes leading to other HIV-1 subtypes being of more globally significant than others (Ranga *et al.*, 2004; Siddappa *et al.*, 2006).

1.2.2.2. Rev

Rev, a 12.76 KDa protein stimulates the production of HIV particles by up regulating the expression of structural genes (*gag*, *pol* and *env*) while down regulating itself and *tat* (Douglas *et al.*, 1997). Rev protein also induces the transition from early phase to late phase of HIV infection (Hanly *et al.*, 1989).

1.2.2.3. Nef

Negative replication factor (Nef) is a 27 kDa protein that retards HIV replication by downregulation of transcription factors natural factor kappa B (NF-κB) and activator protein one (AP-1) (Bandres *et al.*, 1994; Bandres and Ratner, 1994). Nef also induces downregulation of CD4 (Aiken *et al.*, 1994) and HLA class 1 molecules (Collins *et al.*, 1998) from the surface of HIV infected cells which impairs T cell function, thereby helping the virus to invade host immune response (Schwartz *et al.*, 1996). In addition, Nef interferes with T cell activation by binding to various proteins that are involved in intracellular signal transduction pathways (Peter, 1998).

1.2.3. Accessory proteins

1.2.3.1. Vif

Virion infectivity factor (Vif) is a 23 kDa protein which increases the infectivity of the HIV particle. Vif interacts with a cellular protein that belongs to the host's internal cellular defence, known as apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G), which leads to the degradation of the protein (Opi *et al.*, 2007). APOBEC3G is an intracellular antiviral protein that makes virions non-reproductive by deaminating the minus-strand of the viral reverse transcripts introducing numerous G to A mutations. Vif acts by preventing the incorporation of APOBEC3G into newly-formed virus particles.

1.2.3.2. Vpr

Viral protein R (Vpr) is a 15 kDa protein that accelerates the production of HIV proteins by arresting infected cells at the G2 phase of the cell cycle (Jowett *et al.*, 1995), inhibiting cell division by mitosis (Planelles *et al.*, 1995). It also facilitates the nuclear localization of the preintegration complex that is the agglomeration of viral RNA, RT and Integrase proteins which must form in order for the HIV genome to be integrated into the host cell's genome (Miller & Sarver, 1997).

1.2.3.3. Vpu

Viral protein U (Vpu) is a 17kDa protein which is not present in HIV-2, is involved in the assembly of new virus particles and facilitates budding (Deora and Ratner., 2001). The formation of Env-CD4 complexes interferes with viral assembly. Vpu down-modulates CD4 in the endoplasmic reticulum therefore reducing the likelihood of superinfection and is also involved in Env maturation. This reduces the formation of the Env-CD4 complexes. Vpu is not found in virions (Willey *et al.*, 1992).

1.3. Genetic diversity of HIV-1

HIV is divided into two main types, HIV-1 and HIV-2. Phylogenetic analyses have led to the classification of HIV-1 into three genetic groups, namely a major group (group M), an outlier group (group O) and a non-M/non-O group (group N) (Robertson *et al.*, 2000). HIV-1 group M, which is responsible for the global pandemic, is further subdivided into nine different genetic subtypes or clades designated by the letters from A-D, F-H, J and K (Robertson *et al.*, 2000). Subtypes are genetically defined lineages that can be resolved through phylogenetic analysis of the HIV-1 group as well-defined subtypes, or branches in a tree. Currently, strains belonging to the same subtype can differ by up to 20% in the *env* gene and inter-subtype genetic distances can reach up to 35% (Shankarappa *et al.*, 1999). HIV is continually evolving and the epidemic is becoming more complex with recombination.

Recombination occurs frequently and a circulating recombinant form (CRF) is a virus that carries sections of two or more subtypes in a mosaic genome (Fang *et al.*, 2004). A recombinant lineage is designated a CRF when related forms are found in multiple epidemiologically unlinked individuals. To date, 19 CRFs have been identified (Casado *et al.*, 2005). Some of these CRFs are playing an important role in driving regional newly

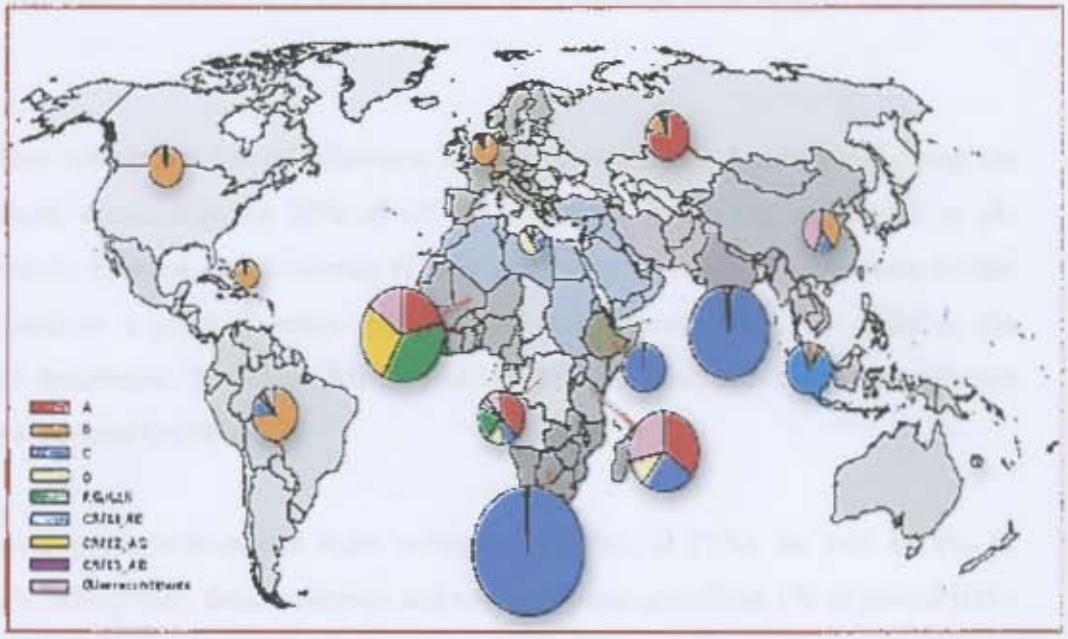
emerging epidemics. For example CRF01_AE and CRF02_AG are dominant CRFs found in Thailand (Xiridou *et al.*, 2007) and West Africa respectively (Fischetti *et al.*, 2004).

HIV-1 subtypes and CRFs are unevenly distributed throughout the world, with the most widespread being subtypes A, B and C, contributing approximately 72% of circulating viruses in 2004 (Hemelaar *et al.*, 2006). Overall, it is estimated that HIV-1 subtypes and CRFs play different roles in the regional sub-epidemics. Subtype C strains, which are predominant in Ethiopia, India, China and southern Africa (Figure 1.4 a), account for 50% of all infections worldwide (Figure 1.4 b) which has increased from 47.2% in 2000 (Osmanov *et al.*, 2002). Subtype A is the predominant subtype in East Africa and accounts for 12% of infections worldwide, B is prevalent in Europe, Brazil, the United States of America, Canada, Australia and Haiti as well as in South African men who have sex with men (van Harmelen *et al.*, 1997) and accounts for 10% of infections worldwide compared to 12.3% from year 2000 data (Osmanov *et al.*, 2002), and D and G are mainly found in central Africa and are responsible for 3% and 6% of worldwide infections, respectively (Figures 1.4 a and b). Subtypes F, H, J and K together are responsible for 0.94% of infections worldwide (Hemelaar *et al.*, 2006).

The CRF01_AE, predominant in Thailand (Xiridou *et al.*, 2007), and CRF02_AG found mainly in Gabon, Ghana and Nigeria (Fischetti *et al.*, 2004), are responsible for 5% of infections worldwide. CRF03_AB only plays a small role in Eastern Europe and Central Asia. Other recombinants accounted for the remaining 8% of infections worldwide. All recombinant forms taken together were therefore responsible for 18% of infections worldwide (Figure 1.4 b) (Hemelaar *et al.*, 2006), illustrating the highly diverse and complex nature of the HIV epidemic.

Of all HIV-1 infections worldwide, 64% are present in sub-Saharan Africa. Fifty-six percent of sub-Saharan African infections are a result of subtype C viruses (Butler *et al.*, 2007). The remaining smaller proportions are caused by subtype A (14%), subtype G (10%), CRF02_AG (7%) and 9% other recombinants (Hemelaar *et al.*, 2006). There are five main sub-regions of sub-Saharan Africa and they also show marked differences in distribution of HIV-1 subtypes and recombinants as well as their global contributions. Southern Africa accounts for 30% of all global infections and Ethiopia contributes 4%. The HIV-1 infections from these two regions of Africa are contributed mainly by subtype C viruses, 98% southern Africa and 99% for Ethiopia. In West Africa, this contributes

(a)



(b)

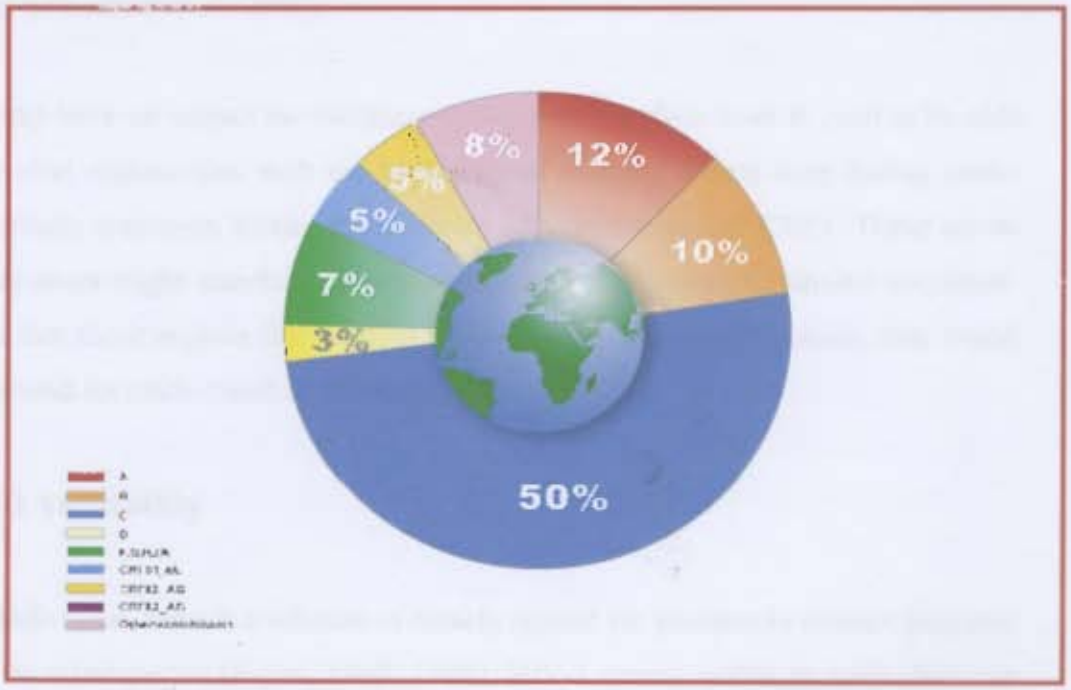


Figure 1.4 (a): Regional distribution of HIV-1 subtypes and recombinants in 2004. Different countries formed regions into which the world was divided. Each region is shaded in different colour. The pie-charts representing the distribution of HIV-1 subtypes and recombinants in each region are superimposed on the regions or connected to the relevant regions by a line. The larger the surface area of the pie-chart, the larger is the number of individuals living with HIV in that particular region. **(b)**: Global distribution of HIV-1 subtypes and recombinants in 2004. The number of infections caused by HIV-1 subtypes and recombinants are represented as a proportion of the global total number of individuals living with HIV-1. The key represents the different HIV-1 subtypes and recombinants. Subtypes F, H, J and K were combined (taken from Hemelaar *et al.*, 2006).

16% of the world's HIV infections, the dominant subtypes are subtype A and CRF02 AG. East Africa accounts for 10% of the world's infections. Overall, the dominant subtypes are A, C and D, each contributing 35%, 25% and 11% of the

infections worldwide respectively and the other 29% coming from unique recombinant forms.

Central Africa contributes 5% of infections worldwide with HIV-1 subtype A being the most prevalent, accounting for 38% of all the infections in the region (Butler *et al.*, 2007). However, subtype D prevalence is also increasing. Overall, the countries in this region all harbour a great diversity of subtypes and recombinants, but differ in the subtype that dominates. In North Africa and the Middle East, the main subtypes are subtype D (47%) and C (29%).

The remaining proportion comes from subtypes A (6%), B (7%), as well as 9% of recombinants. Altogether, these subtypes and recombinants contribute 1% of global HIV-1 infections (Hemelaar *et al.*, 2006).

Diversity may have an impact on vaccine efficacy and therefore there is need to be able to identify viral regions that with the capability of eliciting strong long lasting cross-reactive immune responses across the different HIV subtypes and CRFs. These cross-reactive responses might translate to vaccine induced cross-reactive immune responses. In the case that those regions fail to elicit cross-reactive immune responses, this would suggest the need for clade-matched vaccines.

1.4. HIV-1 variability

Within an individual, there is a mixture of closely related yet genetically distinct genomes referred to as quasispecies (Eigen, 1993; 1996). HIV-1 strains within an individual can differ by as much as 10% in sequence (Shankarappa *et al.*, 1999), while between subtypes (intersubtypes) distances can be as much as 35% in the *env* gene.

1.4.1. Mechanisms of HIV-1 evolution

The enormous variability of HIV-1 is the result of two main mechanisms, mutation and recombination. Forces that drive the evolution of HIV-1 include immune selection pressure, the rapid viral turnover and the availability of cells to infect.

1.4.1.1 Mutation

HIV has a vast evolutionary potential due to a high rate of point mutations, namely $3x10^{-5}$ substitutions per site per generation (Mansky and Temin, 1995). The high error rate of HIV RT results in misincorporation of nucleotides and is a major source of mutations throughout the viral genome and a determinant for rapid viral evolution (Ji *et al.*, 1994; Ji and Loeb, 1994). Rapid evolution of HIV-1 has been shown to correlate with rapid disease progression (Mikhail *et al.*, 2005; Mullins & Jensen, 2006), although it is not clear whether the virus is evolving due to high viral loads or whether it is the evolution resulting in loss of control that lead to high viral loads.

1.4.1.2 Recombination

Genetic recombination is also a source of major variation. Recombination occurs at an average rate of 3 events per genome per round of replication, ranging from 2-20 crossovers (Hu & Temin, 1990). Other studies have found recombination rates of 2.8 per genome per viral replication cycle (Zhuang *et al.*, 2002). Detectable recombination requires infection with more than one virus at the cellular level. Recombination can occur between viruses of the same subtype (intra-subtype) (Philpott *et al.*, 2005) or viruses of different subtypes (inter-subtype). Inter-subtype recombination is a result of the co-packaging of viral genomes from two genetically distinct viruses into a single viral particle in the next cycle of infection. Intra-subtype recombination has been found in 47% of sequences in a subtype C epidemic (Rousseau *et al.*, 2007). Recombination is most frequently identified when it is between subtypes (Charpentier *et al.*, 2006; Yirrell *et al.*, 2002).

1.4.2. Forces driving the variability of HIV-1

Host immune pressure and availability of target cells are both factors driving variability of the virus. The rapid turnover of HIV-1 is also a contributor, as HIV-1 has been shown to produce 10^8 to 10^9 virions per day (Ho *et al.*, 1995; Wei *et al.*, 1995), although it is estimated that 27-66% are defective (Sanchez *et al.*, 1997).

1.4.2.1 Immune selection pressure

The extensive global diversity of HIV is one of the major challenges facing the development of an effective vaccine. It has been suggested that HIV shows stronger effects of positive Darwinian selection (a phenomenon whereby there is a selective pressure forcing change) than any other organism studied so far (Rambaut *et al.*, 2004).

In both HIV and SIV models, there is evidence of positive selection exerted both by neutralizing antibodies (Greenier *et al.*, 2005; Richman *et al.*, 2003; Zhang *et al.*, 1993) and by the host immune cellular immune response (Allen *et al.*, 2000; Barouch *et al.*, 2002; Borrow *et al.*, 1997). Studies on the adaptation of HIV-1 to $CD8^+$ T cell responses at the population level (Moore *et al.*, 2002) are evidence of the role of immune selection pressure in driving the global sequence variability of HIV. The level of this sequence variability is different for different HIV genes. This is because certain mutations in some genes such as *gag*, a structural gene, pose a fitness cost to the virus and this is supported by studies which have shown the reversion of HIV-1 virus to wild type when transmitted to HLA mismatched individuals, suggesting a cost to the viral replicative capacity that may be incurred by acquisition of the relevant escape mutation (Barouch *et al.*, 2005; Fernandez *et al.*, 2005; Friedrich *et al.*, 2004; Matano *et al.*, 2004). However, the *env* gene is a structural gene but there is rapid evolution which is clearly associated with strong autologous neutralizing antibody responses (Liang *et al.*, 2003; Price *et al.*, 1997; Wei *et al.*, 2003). Therefore, greater variability of HIV is created when the virus is trying to escape from immune pressure. The more the viral regions targeted by HIV-specific T cells, the more the virus will mutate in trying to escape from the immune pressure leading to more variability of the virus within an individual as well as within populations when these variants are transmitted.

1.4.2.2 Availability of target cells

During the primary stage of infection and the asymptomatic phase, HIV-1 has been shown to predominantly use the CCR5 coreceptor (Connor *et al.*, 1997). The CCR5 coreceptor utilizing virus that results is referred to as the R5 virus. On the other hand, during the late symptomatic stages of HIV infection, the virus may evolve to show increased tropism for CXCR4. This phenotypic switch has been shown to coincide with the first immunological and clinical signs of diseases progression (Connor *et al.*, 1997). The resulting CXCR4 coreceptor utilizing viral strain is referred to as X4 virus. The availability of different cells expressing the receptors required for HIV-1 infection, that is CCR5 and CXCR4-expressing cells, and the switch from R5 to X4 are evidence for genetic variation (Overbaugh & Bangham, 2001). However, this variation is found in a small gp120 V2 region of the env gene (Jansson *et al.*, 2001). Therefore the availability of CXCR4 co-receptors will select for the X4 viruses that utilizes the CXCR4 coreceptor during the course of HIV infection. However, in some individuals the X4 viruses may retain their ability to use the CCR5 coreceptor and attain dual tropism characteristics

(Doms & Peiper, 1997). Therefore, the more the cells bearing specific receptors used by certain viruses for infection, the more the infection rates and viral turnover of those viruses leading to selection favouring those viruses.

1.5. Immune responses to HIV-1

There is a need to better understand the immune responses that occur during the course of HIV-1 infection to identify immune responses responsible for viral control. For the past 20 years, researchers have been focusing on different aspects of the immune system to determine the immune correlates of HIV control. While earlier studies have looked at the breadth and specificity or magnitude of the immune response and their association with disease outcome, more recent studies have focused on the functionality of the immune response, to identify the qualitative features associated with viral control.

1.5.1. Cellular immune response to HIV-1

Researchers are currently focused on developing vaccine candidates that will stimulate cellular immune responses. Virus-specific $CD8^+$ T cells have been implicated in the control of HIV. HIV-specific $CD8^+$ T lymphocytes have been found in large numbers in a variety of anatomic compartments in both HIV-infected humans and SIV-infected macaques (reviewed in Letvin and Walker 2003). Evidence of the importance of $CD8^+$ T cells in controlling replication has been illustrated in the depletion of $CD8^+$ T cells in macaques infected with SIV which leads to a sharp increase in viremia (Schmitz *et al.*, 1999). Also, a clear temporal association between the expansion of HIV-specific $CD8^+$T cells and viral load reduction was demonstrated in SIV-infected macaque models (Kuroda *et al.*, 1999; Yasutomi *et al.*, 1993) and in humans (Borrow *et al.*, 1994; Koup *et al.*, 1994). Long term non-progressors (LTNPs; those people who take more than ten years to progress to disease) have strong T cell responses to HIV (Gea-Banacloche *et al.*, 2000; Maecker & Maino, 2003; Migueles *et al.*, 2000; Rodes *et al.*, 2004). Further evidence of the importance of $CD8^+$ T cells has been obtained from studies of individuals who are exposed to the virus but remain uninfected (referred to as highly exposed, but persistently seronagative (HEPS) individuals). These individuals were shown to have $CD8^+$ T cell responses to HIV-1, suggesting that the cellular immune response play a role in the prevention of infection (Promadej *et al.*, 2003; Rowland-Jones *et al.*, 1998). However, these individuals must have been transiently infected for them to have HIV-specific $CD8^+$ T cell responses. Thus HIV-specific $CD8^+$ T cells appear to play a role in

controlling HIV infection in some individuals, although viral replication is not fully contained in most infected people.

The acquisition of the capacity to stimulate $CD8^+$ T cells by antigen presenting cells has been shown to involve $CD4^+$ T cell help (Xiang *et al.*, 2005). Furthermore, $CD4^+$ T cells are necessary for clonal expansion of $CD8^+$ T cells and their differentiation into cytotoxic T lymphocytes (Janssen *et al.*, 2003), as well as for maintaining memory of $CD8^+$ T cells after acute infection (Sun and Bevan, 2003). The magnitude of $CD4^+$ T cell proliferation and IL-2 production by $CD4^+$ T cells has been shown to correlate with the clinical status of HIV-infected humans and SIV- or SHIV-infected monkeys (Rosenberg *et al.*, 1997; McKay *et al.*, 2003). Thus HIV-specific $CD4^+$ T cell responses are also important for viral control.

The HIV regions targeted by the cellular immune response will help in choosing immunogens for vaccine design. Most HIV-infected individuals mount robust T cell responses to HIV (Addo *et al.*, 2003; Betts *et al.*, 2001). All HIV proteins are immunogenic and HIV-infected individuals have responses in the order Nef > Gag > Pol > Env > Vif > Rev > Vpr > Tat > Vpu (Masemola *et al.*, 2004). Generally, studies have shown that Gag and Nef are the most immunodominant HIV proteins. Individuals have T cell responses to a median of 14 epitopes (range 2-42) across the HIV proteome (Addo *et al.*, 2003).

The identification of immune correlates of viral control is important in HIV vaccine development. In an effort to identify immune correlates of HIV control, studies have looked at the magnitude and breadth of HIV-specific T cell responses and their effect on either the number of $CD4^+$ T cells, rate of $CD4^+$ T cell decline or viral load (Addo *et al.*, 2003; Masemola *et al.*, 2004; Peretz *et al.*, 2005). In one study, fifty seven individuals were screened for HIV-specific T cell responses using a matrix of 504 overlapping peptides spanning the entire expressed HIV genome using an ELISpot assay (Addo *et al.*, 2003). In this study, neither the breadth nor the magnitude of total HIV-specific T cells correlated with viral load. In another study on subtype C-infected individuals, the breadth of the T cell responses did not correlate with viral load (Masemola *et al.*, 2004). However, the magnitude of HIV-specific T cells in these individuals had a weak positive correlation with viral load. This was also found in another study of HIV-infected children (Buseyne *et al.*, 2002), implying that the magnitude of T cell recognition is not the

correlate of immune control of the virus. These results are further supported by other studies which demonstrated that there is no significant difference in the breadth and magnitude of HIV-specific T cell responses among typical, fast and slow progressors (Peretz *et al.* 2005), and that the overall frequencies of HIV-specific T cell responses do not correlate with viral load and are therefore not the determinant of immune-mediated protection in HIV infection (Betts *et al.*, 2001).

The majority of the aforementioned studies measured a single functional of the cellular immune response, namely the production of IFN-γ. It is of importance to identify the functional nature of T cells that are responsible for HIV control, so that vaccine immunogens can be designed to induce those functions. T cells have the capacity to secrete multiple cytokines or chemokines, to degranulate and to proliferate. A recent study found that LTNPs possess a higher proportion of $CD8^+$ HIV-specific T cells positive for four and five functions than progressors (Betts *et al.*, 2006). Thus multiple immune functions by HIV-specific T cells may be important for viral control.

An important finding of several studies on the cellular immune response to HIV is the importance of the Gag protein. A number of studies, performed in individuals of diverse ethnicities, have suggested that Gag-specific T cell responses could be especially important in viral control (Ogg *et al.*, 1998; Buseyne *et al.*, 2002; Edwards *et al.*, 2002; Novitsky *et al.*, 2002; Masemola *et al.*, 2004; Ramduth *et al.*, 2005). In a study of chronically infected individuals, $CD8^+$ T cell responses were equally distributed among Gag, Pol and the regulatory and accessory proteins, but with Gag being a dominant target for $CD4^+$ T cells (Ramduth *et al.*, 2005). In this study, no consistent relationship was found between the magnitude of virus-specific $CD8^+$ or $CD4^+$ T cell responses and viral load. However, gag responses were associated with lower virus loads. Another study showed that the magnitude of functional $CD8^+$ T cell response to the Gag protein is inversely correlated with viral load (Edwards *et al.*, 2002). These results are further supported by studies which have shown that it is not the recognition of Gag proteins that is associated with control of viremia but is the preferential targeting of Gag that was significantly associated with viral control (Masemola *et al.*, 2004; Zuniga *et al.*, 2006). Other studies have further dissected the Gag response into its three main regions and found that the p24 region of Gag is the most immunodominant region of this protein (Geldmacher *et al.*, 2007). A large cross sectional study further confirmed these results. The breadth of Gag-specific responses was found to be associated with low viremia,

while that of Env-specific responses with high viremia (Kiepiela *et al.*, 2007). In this study it was the breadth of Gag-specific response that was associated with decreasing viremia and this correlation was not associated with the HLA type of the individuals.

Thus, specificity and function of the immune response may be more important than overall magnitude and breadth. It is thus important to have a clear understanding of cellular immune responses against HIV, as this will make important contributions to HIV vaccine development.

1.5.2. Host genetic factors –The role of the Human Leukocyte Antigen (HLA) in the pathogenesis of HIV-1 infection

The task of designing an effective vaccine is made difficult by both the genetic diversity of the virus as well as the diverse human leukocyte antigen (HLA) backgrounds of individuals in different populations. An important consideration in understanding cellular immune responses to HIV-1 infection is the role played by HLA molecules, through which $CD8^+$ T cells and $CD4^+$ T cells recognize viral epitopes.

HLA class I molecules are involved in the presentation of immunogenic peptides to $CD8^+$ T cells while HLA class II molecules present immunogenic peptides to $CD4^+$ T cells (Gebe *et al.*, 2002). HLA class I molecules involved in peptide presentation are classified into HLA-A, -B, -C, while as HLA class II are classified into HLA-DP, -DQ and –DR. HLA class III molecules are unrelated to peptide presentation. The type of peptide fragment that binds to a particular HLA is a function of the chemical nature of the groove for that specific HLA molecule and the sequence of the peptide (Reche & Reinherz, 2003).

Different populations and ethnicities express different HLA alleles (Ferrari *et al.*, 2004; Wadee & Dunn, 1991; Watkins *et al.*, 1992; reviewed in Stephens, 2005). Even in individuals of the same ethnicity, particular HLA alleles expressed differ among individuals (du Toit *et al.*, 1990a; 1990b). An allele that is common in one population may be rare in another population. Some alleles are limited to particular ethnic populations, while others are widely shared among ethnically distinct populations. Previous studies have shown that HLA-A30, -A68, -B15, -B42 and –B58 are commonly found in Zulu and Xhosa South Africans but are less frequent in Caucasians (Marsh, 2000). However, HLA-A2 and B44 appear relatively frequent in the Caucasian

population and less frequent in The Zulu and Xhosa people. HLA-A11 is a common HLA allele in coloured, but virtually absent in Xhosa people (du Toit *et al.*, 1987). Interestingly, HLA-A11 is also common in south-East Asia and specifically Thailand (Lynch *et al.*, 1998). Another example is the allele HLA-A36 that is found only among individuals of African descent. HLA-A2 is among the most common HLA types in all populations (Chandanayingyong *et al.*, 1997).

The extent to which polymorphism occurs is different for different HLA molecules. For example, within the HLA class I molecules, polymorphism is more common among HLA-B alleles than HLA-A or HLA-C alleles. There are 563 HLA-B alleles, 309 HLA-A alleles and 167 HLA-C alleles described, showing that the HLA-B locus is diversifying more than the other loci (Kiepiela *et al.*, 2004).

As HLA class I molecules bind fragments of viral proteins and present these to immune cells to initiate responses, the particular fragment of a virus that is immunogenic for T cells and the magnitude of virus-specific T cell responses are determined in part by the HLA class I molecule expressed in an individual. For example, the SLYNTVATL (SL9) fragment of HIV-1 Gag binds to the HLA-A2 molecule and presents it to immune cells, resulting in a relatively reproducible high-frequency Gag-specific T cell response in HLA-A2-positive individuals (Schmitt-Haendle *et al.*, 2005).

There are now data that certain HLA types, including HLA B57 (Altfeld *et al.*, 2003; Stewart-Jones *et al.*, 2005), B27 (Stephens, 2005), B5801 (Gao *et al.*, 2001; Migueles *et al.*, 2000) and B63 (Frahm *et al.*, 2005) are associated with better disease outcome (Altfeld *et al.*, 2006). It may be that these HLA molecules are favouring the selection of variants that have escaped T cell immune pressure and have lower replication capacity (Martinez-Picado *et al.*, 2006). On the other hand, HLA B35, B5802 and Cw*04 have been shown to negatively affect the outcome of disease (Walker & Korber, 2001). This might be due to HLA molecules favouring escape mutants with higher replication capacity resulting in higher viral set points.

Studies have shown that there is differential contribution of different HLA molecules to the total anti-HIV cellular immune response and HLA-B molecules were shown to play a dominant role in shaping the co-evolution of HIV-1 and HLA molecules (Kiepiela *et al.*, 2004). Kiepiela *et al.* (2004) study also suggested the dominant role of HLA-B molecules

in the containment of HIV. This locus was shown to contribute the most to the total cellular immune responses against HIV. Those viral genes whose peptides are presented to T cells have strong immune pressure exerted on them. There is a strong selective pressure for survival of HIV mutants that escape the T cell response and those mutants that are stable are transmitted and accumulate in the population (Goulder & Watkins, 2004; Leslie *et al.*, 2005). There are studies which suggest that HIV adapts to the most frequent HLA alleles in a population and this provides selective advantage for those individuals who express rare alleles (Trachtenberg *et al.*, 2003).

Different HLA molecules can present similar or identical viral epitopes despite their polymorphism, and have therefore been grouped into at least nine major HLA supertypes, based on analysis and subsequent clustering of their peptide binding repertoire (Doytchinova *et al.*, 2004; Kangueane *et al.*, 2005; Sette & Sidney, 1999). This may be important for candidate vaccines which do not contain whole proteins but just parts of genes or epitopes in order to make predictions on whether they will be effective in particular populations expressing particular HLA types.

Given the extensive polymorphism known to exist within HLA and even more genetic variation to be revealed as more populations are studied, it may be important to identify those viral epitopes that can be recognized by individuals with different HLA backgrounds and which are conserved across HIV-1 subtypes. These epitopes may be important for inclusion in candidate vaccines.

1.5.3. HIV Cross-clade cellular immune responses in HIV-1 infected individuals

Given the genetic diversity of HIV and its rapid evolution rate, the selection of suitable antigens and epitope variants is important in the design of an effective vaccine. One way to overcome this obstacle is to design HIV immunogens able to induce broadly cross-reactive cellular immune responses that can recognize a wide selection of different viral quasispecies. An immune response in an individual infected or vaccinated with one HIV-1 subtype, that is cross-reactive to a different HIV subtype, will be referred to here as a 'cross-clade' immune response. For a vaccine to be effective, T cell responses must successfully combat a diverse array of viral variants, including within and between viral genetic clades or subtypes.

Studies conducted thus far have detected high frequencies of HIV-specific T cells that clearly have the ability to cross-recognize different HIV-1 strains and subtypes. However, in most early studies, cross-recognition was either assessed for a limited number of selected epitopes (Cao *et al.*, 2000; Fukada *et al.*, 2002; Rutebemberwa *et al.*, 2004), or at the protein level in chromium release assays using cells infected with vaccinia virus constructs expressing whole HIV proteins (Buseyne *et al.*, 1998; Cao *et al.*, 1997; Ferrari *et al.*, 1997). These studies were thus limited because they could not determine the degree of cross-reactivity of HIV-specific T cells at the single epitope level. More recent studies have looked at cross-clade immune responses using sets of overlapping peptides covering whole functional proteins or the entire expressed HIV genome, and have been more informative. For cross-clade studies to be informative in vaccine design, it is important also that they determine the degree to which different viral subtypes are cross-recognized in individuals infected with different subtypes, that is the frequency of recognition of different HIV subtypes in different populations with different HLA backgrounds.

In one study, cellular immune responses to HIV clades A, B and C were assessed in a cohort of 250 subjects infected with these different HIV-1 subtypes from Brazil, Malawi, South Africa, Thailand and the United States (Coplan *et al.*, 2005). In this study, cross-clade cellular immune responses were substantial for Nef and lower for Gag among these subtypes, with the fraction of homologous subtype's responses to the heterelogous subtype's responses (referred to as cross-clade reactivity ratios) being 0.97 and 0.67, respectively. These reactivity ratios were defined as the fraction of cellular immune response to a heterelogous subtype (the subtype not responsible for infecting an individual), compared to a homologous subtype (the subtype responsible for infecting the same individual). This difference in reactivity ratios between these proteins was found to be significant (Coplan *et al.*, 2005). These results were further supported in a larger cohort of 363 (Gupta *et al.*, 2006).

The degree of cross-clade HIV-specific T cell responses to specific epitopes across the entire HIV proteome was investigated by Yu *et al* (2005). Twenty-seven chronically clade B-infected individuals, were tested for responses to peptides based on subtypes A, B and C. Although there was preferential recognition of the peptides based on the infecting subtype (subtype B), there was no significant difference in the total magnitude and breadth of HIV-specific T cell responses to peptides spanning the entire HIV consensus proteomes among these major HIV-1 subtypes. Interestingly, a significant

proportion (34%) of detected HIV-specific T cells showed cross-reactivity among the three HIV-1 subtypes. In addition, broadly cross-reactive T cell responses were preferentially directed against those viral regions with low intra-clade entropy and simultaneously high inter-clade homology such as Gag, Nef and Pol, with Gag being the most cross-reactive protein (Yu *et al.*, 2005).

Sequences that are representative of all the HIV-1 subtypes (referred to as consensus or ancestral sequences) can be generated using phylogenetic methods. A recent study assessed cross–clade immune responses to consensus subtype A, B,C as well as group M, ancestral M and virus based subtype B HXB2 Gag peptides in 43 subtype B-infected US subjects and 13 subtype C-infected Zambians subjects (Bansal *et al.*, 2006). This study was an effort to determine if minimizing the genetic distance between HIV sequences infecting individuals and HIV sequences includes in vaccine candidates would have an impact on cross-clade responses. The study demonstrated sequences that are representative of all the HIV-1 group M subtypes can be used to evaluate HIV Gag-specific responses. However, this idea as a vaccine concept needs to be tested in primate (human or non-human) vaccine recipients to determine whether responses elicited by a consensus immunogens are more cross-reactive than those seen by a single viral isolate (Bansal *et al.*, 2006).

Bioinformatics tools can be used to look for reasons for lack of cross-clade recognition to some peptides. In these theoretical approaches, combinatorial libraries were used to derive HIV-1 Gag p17 SL9 epitope mimics (Boggiano *et al.*, 2005). Laboratory assessment of responses to these mimics found that those mimics that have amino acid changes at the conserved F pocket anchor residues are less frequently cross-recognized. In addition, changes in T cell receptor contact sites have been shown to abrogate the recognition of peptides by HIV-specific T cells (Malhotra *et al.*, 2007). Peptide variations outside the HLA anchor residues of the epitopes have been shown to have less impact on cross-clade recognition (Geels *et al.*, 2005).

Therefore, HIV-specific T cells have been shown to frequently recognize peptides based viral subtypes that are different from the infecting virus. Studies have shown that those viral regions that are conserved among the different HIV-1 subtypes such as the *gag* gene are more frequently cross recognized than those that are less conserved. Thus cross-clade T cell activity is determined by two important factors: viral sequence diversity and the

HLA class I profile of the infected or vaccinated host. The generation of an HIV vaccine designed to elicit HIV-specific T cell activity must take these factors into account to prime effective intra- and inter-clade T cell reactivity.

1.6. HIV Vaccine approaches and vaccine-induced cross-clade immune responses

HIV has proved a difficult pathogen to develop a vaccine against. The development of such a vaccine has encountered a number of scientific challenges, including an incomplete understanding of the immune correlates of protection, the limitations in animal challenge models, and the significance of genetic and immunologic variability of HIV strains for potential vaccine candidates. It is unclear whether viral diversity together with HLA polymorphisms found in different populations will limit broadly reactive cellular responses which are an important concern for developing a globally effective vaccine against HIV. There are several candidate HIV vaccines employing different approaches, which are currently in development, either in clinical (human) trials or in preclinical development in non-human primates.

It is generally accepted that an effective HIV vaccine will have to stimulate both neutralizing antibodies and cell-mediated immune responses in blood and at mucosal sites (McMichael, 2003; McMichael & Hanke, 2003). Most antibodies produced in response to HIV infection afford little if any control of viremia, appearing to select for virus escape mutants (Richman *et al.*, 2003; Wei *et al.*, 1995). However, passive immunization experiments have shown that neutralizing monoclonal antibodies can effectively protect from infection (Mascola *et al.*, 2005b). Despite numerous approaches to elicit neutralizing antibodies (Calarota & Weiner, 2003) there is still no immunogen that can elicit neutralizing antibodies which are capable of effectively neutralizing primary isolates (Burton *et al.*, 2004; Garber *et al.*, 2004). If neutralizing, the antibodies require the induction of high titres difficult to achieve through vaccination (Mascola *et al.*, 2005a; Mascola *et al.*, 2005b). Due to difficulties in developing a vaccine that elicits neutralizing antibodies, the field has focused on vaccines aimed at eliciting T cell responses.

A few promising approaches capable of inducing high levels of circulating T cell responses have emerged, including vaccines based on Adenovirus 5 vectors, naked DNA, and poxviruses (http://www.iavireport.org/Issues/CurrentIAVIReport.asp). However, the

disadvantage of T cell vaccines is that, they may not prevent the first wave of cell-free HIV from infecting host cells (Hanke, 2004). These vaccines may therefore primarily result in ameliorating disease, rather than preventing infection.

There have been several approaches to design vaccine immunogens which are most likely to elicit cross-clade immune responses. One of these approaches is the development of vaccines based on a single HIV-1 subtype representative of the dominant virus circulating in a specific geographical region. Examples of this include the development of several A- and C-subtype vaccines, as well as CRF_01 AE vaccine reagents (Graham *et al.*, 2006; Schultz & Bradac, 2001). The subtype C multigene SAAVI DNA-C vaccine, which expresses *gag*, *RT*, *tat*, *nef* and truncated *env*, selected vaccines genes from recently infected individuals based on closest to a South African consensus sequence (Burgers *et al.*, 2006). This vaccine is targeted to the southern Africa region where subtype C predominates.

An alternative immunogen approach is based on phylogenetics, rather than using actual virus from within the population. In this approach, sequences are based on a consensus sequence or an ancestral sequence which will theoretically have more epitopes in common than an actual sequence. Such sequences have the advantage of being central and most similar to currently circulating strains of interest and may have enhanced potential for eliciting cross-reactive responses (Gaschen *et al.*, 2002). An example of such a vaccine is the clade B-based Adenovirus serotype 5 HIV-1 Gag/Pol/Nef vaccine produced by Merck, currently being tested in adult South Africans. The vaccine is based on the subtype B CAM-1 strain sequence.

Another approach to developing a broadly cross-reactive vaccine is the multiclade approach, whereby constructs expressing HIV genes from different subtypes are combined. Using this approach, there is potential of individuals infected with different HIV-1 subtypes to recognize at least one of the immunogens used in the vaccine as it might correspond to the subtype with which they are infected. An example of the multi-clade/subtype vaccines in Phase II studies is the DNA prime-Adenovirus 5 boost vaccine which expresses clade B *gag*, *pol*, *nef*; as well as clade A, B, and C *env* (http://chi.ucsf.edu/vaccines/ vaccines? page=vc-01-01). Positive results have been obtained during Phase I trials of this vaccine formulation (Graham *et al.*, 2006). The multiclade Env immunogens have been shown to elicit a greater breadth of immune

responses than single subtype-based immunogens in rhesus macaques (Seaman *et al.*, 2005).

Identification of only the immunogenic regions of the HIV proteome forms part of another approach of epitope-based vaccines in which CD8 epitopes are combined to form a vaccine. An example of this type of vaccine is the DNA- and Modified virus Ankara (MVA)-vectored candidate HIV vaccine expressing HIV-1 subtype A-derived p24/p17 Gag fused to a string of HLA class I epitopes, the HIVA vaccine. This vaccine was targeted for East Africa as HIV-1 subtype A is the dominant circulating subtype in this region (Hanke *et al.*, 2002). Poor immunogenicity of the vaccine however halted further clinical testing (Hanke *et al.*, 2007).

In order to be able to know which vaccine formulation works best, it will be necessary to perform vaccine efficacy trials. However, vaccine trials are lengthy, clues to whether immune responses generated will be cross-reactive can be obtained by testing for immune responses to HIV peptides included in the candidate vaccines using PBMCs from HIV-infected individuals.

1.7. Aim of the thesis

South Africa is faced with an enormous epidemic of HIV/AIDS and an effective vaccine is urgently needed to prevent new infections. HIV-1 is a highly variable virus and it is impractical to duplicate vaccine design efforts using country-specific strains for every nation and region that needs a vaccine. In addition, it is difficult to change the strains used during the long course of vaccine development, from initial concept to human trial. There are vaccines in advanced stages of development and testing that contain different HIV-1 subtypes. It is important to generate data that explores intra- and cross-clade T cell immune responses to vaccine immunogens included in vaccines. As host HLA background is inextricably linked to cross-clade recognition, it is important to perform these studies in a South African population since results cannot be extrapolated across populations. The data could then be used to assess the degree of cross-reactivity in a population where advanced phase vaccine trials are likely to take place in the future.

The aim of the study reported in this thesis was to measure cross-clade immune responses to predict whether a vaccine based on one subtype will be effective in a population where a different subtype is circulating. To perform this study we obtained HIV-1 Gag peptides based on sequences currently included in vaccines, including a South African subtype C (strain Du422), a Chinese subtype C strain, the subtypes B CAM-1 strain sequence and sequences from subtypes A and D. The peptides were obtained from the International AIDS Vaccine Initiative (IAVI) (subtypes A, $C_{S.A}$, C_{CH} and D) and The National Institute of Health (NIH) Reference Reagent Repository (subtype B) and are current or potential future sequences included in candidate HIV vaccines

1.7.1. Specific objectives

1. To characterize the sequence of the infecting HIV-1 strain in HIV-1 infected subjects
2. To determine intra-clade HIV-specific T cell immune response using HIV Gag peptides based on HIV Gag immunogens currently incorporated in HIV-1 subtype C vaccines, namely
   i. a Chinese subtype C isolate
   ii. a South African subtype C (isolate Du422)

3. To assess the degree of cross-clade HIV-specific T cell immune responses using peptides based on HIV Gag immunogens which are incorporated into vaccine candidates or may form part of future vaccine candidates, namely
   i. a subtype A sequence
   ii. a subtype B sequence
   iii. a subtype D sequence

CHAPTER 2

Genetic characterization of HIV-1 *gag* sequences

2.1 INTRODUCTION

2.1.1 HIV-1 Gag as a vaccine immunogen

The development of a safe, globally effective and affordable vaccine offers the best hope for the future control of the HIV pandemic. One of the major challenges in developing such a vaccine is the high degree of genetic diversity the virus exhibits. This high variation among gene sequences of HIV viruses belonging to different lineages is fuelled by high mutation, recombination and replication rates and driven by host immune pressures. The significance of HIV genetic variation in vaccine efficacy remains unresolved, but there is some indication that it might play an important role (Gaschen *et al.*, 2002).

It is important for vaccine development, to know which regions of the viral genome, if targeted by the immune system, will result in the control of the disease. The best vaccine formulation should elicit both humoral and cell mediated immunity which is cross-reactive between the different HIV variants and subtypes. Studies have shown that preferential targeting of the HIV Gag protein is associated with better control of the virus (Masemola *et al.*, 2004; Zuniga *et al.*, 2006). A more recent study showed that broader Gag responses are associated with viral control (Geldmacher *et al.*, 2006; Kiepiela *et al.*, 2007). Gag is therefore an important region to include in candidate vaccines. In addition, Gag is important to studies investigating cross-clade responses as it is rich in T cell epitopes which are commonly targeted by the host cellular immune response (Addo *et al.*, 2003).

HIV *pol* is the most conserved HIV gene (>90%) across HIV-1 subtypes. *Nef* is also relatively conserved (80%) across subtypes A, B and C. However, *env* is more variable (>20%) even within a single subtype. HIV *gag* is relatively well conserved (85%) among subtypes A, B and C (http://hiv-web.lanl.gov). It is divided into three main regions which encode three proteins, namely p17, p24 and p15. The p24 region of *gag* is highly conserved, while the p17 and p15 regions are more variable. This might be due to structural constraints, that is, mutations within the p24 might results in replicative defective or unfit viruses. However, there are well conserved motifs within the more variable p15 region of *gag*. These conserved motifs are encompassing the Vpr binding regions if HIV *gag* and are found in the p6 region of p15. This nature of *gag*, containing both conserved and variable regions, will enable the comparison of cross-clade T cell mediated responses elicited by either variable or conserved regions of HIV. For the purpose of eliciting cross-reactive T cell immune

responses, the conserved regions of the protein are expected to make a major contribution, since results from previous studies suggest that cross-reactive HIV-specific T cells recognize regions with lower entropy and higher inter-clade homology (Yu *et al.*, 2005). It is also important to know the kind of mutations leading to the abrogation of cross-reactive T cell recognition, hence the importance of having gene sequence data.

Most T cell vaccines currently in development contain *gag*. Together, HIV-1 subtypes A, B, C and D account for approximately 72% of infections worldwide. It is therefore important to genetically characterize the HIV *gag* gene as it is contained in several vaccine immunogens to relate this sequence data with intra- and inter-clade T cell responses detected.

2.1.2 Aim of the chapter

This study aims to characterize HIV-1 *gag* sequences from subtype-C infected individuals from South Africa who will be investigated for cross-clade responses (chapter three).

2.1.3 Specific objectives

The specific objectives of this chapter are to

i. Determine the sequence of the infecting virus and investigate the phylogenetic relatedness between study sequences and known sequences
ii. Compare the infecting viral sequences to the ELISpot peptide reagents used in cross-clade immune study. The subtypes A, B, $C_{S.A}$, C_{CH} and D Gag peptides used to investigate immune responses are based on HIV isolate sequences included in current or planned HIV vaccine candidates. Peptides were provided by the International AIDS Vaccine Initiative, except for the subtype B CAM-1 strain peptides which were obtained from the National Institute of Health AIDS Research and Reference Reagent Repository.

2.2 MATERIALS AND METHODS

2.2.1 Characteristics of study subjects and sample collection

Forty asymptomatic HIV infected individuals were recruited from each of the following sites: 20 from the Perinatal HIV Research Unit at Chris Hani Baragwaneth Hospital, Soweto; and 20 from clinics of the Desmond Tutu HIV Centre in Gugulethu, Cape Town. All participants signed an informed consent form prior to study enrolment. The University of Cape Town Research Ethics Committee approved the study protocol (Ref Number 211/2004). The criteria for patient inclusion was a CD4 count > 350 cells/μl with no overt evidence or history of opportunistic infections. Sixty ml of blood were obtained from each individual by venipuncture in Acid-Citrate-Dextrose (ACD) tubes; Peripheral Blood mononuclear Cells (PBMCs) were isolated using standard Ficoll-Hypaque density gradient centrifugation and cryopreserved for immunological assays described in Chapter three. Plasma was also isolated from the gradient and stored at -70^{0}C. A further 5 ml Ethylene-Diamine-Tetra-Acetic acid (EDTA) blood was obtained from the same individuals, plasma isolated and stored at -70^{0}C for viral sequencing.

2.2.2 RNA extraction

The QIAamp® Viral RNA Mini Kit was used for RNA extraction (QIAamp® Viral RNA Mini Kit, QIAGEN, Valencia, CA). The sample is first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample is loaded onto the QIAamp spin column. The RNA binds to the membrane, and contaminants are washed away in two steps using two different buffers containing sodium azide and ethanol. In the first step the column is centrifuged with buffer AW1 and at 6000 x g (8000 rpm) for one minute, and then with the second wash buffer AW2, at 20 000 x *g* (14000 rpm) in a micro centrifuge (Eppendorf 22331, AG Hamberg, Germany). RNA is eluted by centrifugation at 6000x g (8000 rpm) in an RNase-free buffer. Plasma sample (140μl) was used to extract RNA which was subsequently eluted in 60μl RNase-free buffer according to manufacturer's instructions (Appendix A1). The RNA was either aliquoted into 10μl aliquots and stored at -80^{0}C, or used directly for complementary deoxyribonucleic acid (cDNA) synthesis.

2.2.3 Full length *gag* cDNA synthesis

HIV-1 *gag* cDNA was generated using the Invitrogen Thermoscript™ RT-PCR System (Invitrogen, GmbH, Karlsruhe, Germany). The system uses Thermoscript RT, an avian RNase H-minus reverse transcriptase enzyme to generate DNA using RNA as template. One microlitre of 10mM dNTP mixture (dATP, dTTP, dCTP and dGTP) and 1µl (10pmol, 2µM) of *gag*-specific primer, *Gag* D reverse (5' AAT TCC TCC TAT CAT TTT TGG 3') were mixed with 4µl of HIV-1 RNA and heated at 65^0C for 5 minutes and then cooled on ice. The synthesis of cDNA was initiated by the addition of 4µl of RT master mix (Table 2.1) and incubating at 55^0C for one hour. The reaction was terminated by heating at 85^0C for 5 minutes. RNA template was then removed by adding 0.5µl of 2U of *E.coli* RNase H supplied with the kit and incubating at 37^0C for 20 minutes. All cDNA synthesis steps were carried out using the GeneAmp® PCR system (GeneAmp® PCR system 2700, Singapore).

Table 2.1 cDNA synthesis reagents and volumes for RT PCR amplification

cDNA synthesis reagent	Volume for 1x reaction (µl)
5x cDNA synthesis Buffer	2.0
0.1 M DTT	0.5
Rnase-Out (40U/µl)	0.5
RT (15U/µl)	0.5
Distilled water (dH_2O)	0.5
Total	4.0

2.2.4 Polymerase chain reaction (PCR) and Agarose gel electrophoresis

Nested polymerase chain reaction (PCR) was used to amplify *gag* sequences, whereby two sets of primers are used in two rounds of polymerase chain reactions with the second PCR product being shorter than the first one. This is performed to have shorter fragments for sequencing.

2.2.4.1 First round PCR

cDNA from the RT step was amplified in a first round PCR using gene-specific primers, *Gag* D forward 5' TCT CTA GCA GTG GCG CCC G 3' (HXB2 626-644) and *Gag* D reverse 5' AAT TCC TCC TAT CAT TTT TGG 3' (HXB2 2402-2382) where the numbers are the position of the nucleotide sequences of the primers relative to the HXB2 nucleotide sequence where the primers bind (Bredell *et al.*, 2007). The composition of the reaction and cycling conditions are shown in Table 2.3 and Table 2.4 respectively.

Table 2.2 PCR reagents and volumes for the 1st round PCR amplification

PCR reagent	1st round 1x reaction volume (µl)
10x Buffer	5
10mM Mg	4
10mM dNTPs	6
Primer *gag* D reverse	1
Primer *gag* D forward	1
SuperTherm	0.125
Distilled water	27.875
cDNA template	5
Total	50

Table 2.3 PCR cycling conditions

Cycling condition		Time and Temperature (^{0} C)
(denaturing)		2 minutes (94^0 C)
	1 cycle	
(denaturing)		1 minute (94^0 C)
(annealing step)		1 minute (55^0 C)
(extension step)		1 minute (72^0 C)
	3 cycles	
(denaturing)		15 seconds (94^0 C)
(annealing step)		45 seconds (55^0 C)
(extension step)		1 minute (72^0 C)
	32 cycles	
(extension step)		7 minutes (72^0 C)
	1 cycle	
-END		-END (4^0 C)

2.2.4.2 Second round Polymerase chain reaction

Three *gag* regions (A, B and C) were amplified separately in a nested PCR. Five µl of DNA from the first round PCR was added to a second round master mix, whose composition is shown in Table 2.4 below. The remainder of the first round product was stored at -20^{0}C. Primers used and their binding positions according to the HXB2 numbering positions were:

A forward, 5' CTC TCG ACG CAG GAC TCG GCT T 3' HXB2 683-704;

A reverse, 5' ACA TCG GTA TCA CTT CTG GGC T 3' HXB2 1303-1282;

B forward, 5' CCA TAT CAC CTA GAA CTT TGA AT 3' HXB2 1226-1248;

B reverse, 5' CTC CCT GAC ATG CTG TCA TCA T 3' HXB2 1846-1825;

C forward, 5' CCT TGT TGG TCC AAA ATG CGA 3' HXB2 1748-1768;

C reverse, 5' TCT TCT AAT ACT GTA TCA TCT GC 3' HXB2 2356-2334.

Table 2.4 PCR reagents and volumes for the 2nd round PCR amplification

PCR reagent	Fragment A	Fragment B	Fragment C (volumes, µl)
10x Buffer	5	5	5
10mM Mg	2	6	4
10mM dNTPs	4	4	4
Primer *gag* reverse	1	1	1
Primer *gag* forward	1	1	1
SuperTherm	0.125	0.125	0.125
Distilled water	31.875	27.875	31.875
cDNA template	5	5	5
Total	50	50	50

Table 2.5 PCR cycling conditions

Cycling condition		Time and Temperature (^{0} C)
(denaturing)		2 minutes (94^0 C)
	1 cycle	
(denaturing)		1 minute (94^0 C)
(annealing step)		1 minute (55^0 C)
(extension step)		1 minute (72^0 C)
	30 cycles	
(extension step)		7 minutes (72^0 C)
	1 cycle	
-END		-END (4^0 C)

Ten µl of the second round PCR was used for Agarose gel electrophoresis as described in the next section, and the remaining 40µl was purified for sequencing using the PCR product purification kit as described in section 2.2.5.1.

2.2.4.3 Agarose gel electrophoresis

The constant negative charge on nucleic acids allows them to be separated in an electric field in agarose gel electrophoresis based on their size. Agarose gel electrophoresis was used to visualize amplicons using horizontal gel electrophoresis apparatus (Stratagene, La Jolla, USA). A 1% agarose gel was prepared by melting agarose powder (Agarose Di LE, Hispanager, Burgos, Spain) in 10 x TBE buffer (Appendix B3). Ten µl of ethidium bromide

(10 mg/ml), a fluorescent dye used for staining nucleic acids was added to a final concentration of 5%. The gel was left for 30 minutes which was sufficient for it to cool and set, after which, it was submerged in 1 x TBE buffer before loading the samples into the wells. Ten µl of DNA sample (PCR product) was mixed with 3µl (~16ng) of 0.25% bromophenol blue, an agarose gel electrophoresis loading dye that is used to track the migration of DNA fragments in an agarose gel, and then loaded into agarose gel wells. Three µl of DNA molecular weight marker VI at a final concentration of 0.75µg shown in Figure A1, Appendix A4 (Roche, GmbH, Mannheim, Germany) diluted in 7µl of distilled water was loaded on the gel in order to determine the size and quantity of the PCR amplicons. The gel was run at 100 V for 60 minutes, sufficient for effective band separation. Visualization of DNA fragments was done using a UVP trans-illuminator (UVP, San Gabriel, California, USA) at 256 nm wavelength and then photographed with a Kodak ds ID Electrophoresis and Documentation Analysis System 120, v 2.0.3 computerized gel imager and software (Kodak ds ID digital science, v2.0.3).

2.2.5 Purification and quantification of PCR amplified *gag* molecules

2.2.5.1 Purification

The QIAquick^{R} Spin Purification Procedure was used for the PCR product purification process (QIAquick^{R} PCR purification Kit, QIAGEN, Valencia, CA). The process involves binding of DNA to the silica-gel membrane in the presence of a high salt concentration, followed by washing away of impurities using an ethanol containing buffer (buffer PE), and centrifugation and then elution of pure DNA with Tris-Cl buffer (10mM, pH 8.5). Forty µl of sample DNA were purified and eluted in 50µl of elution buffer according to manufacturer's instructions (Appendix A2).

2.2.5.2 Quantification

The DNA was quantified following loading of a known amount onto an Agarose gel and comparing the relative intensity to Molecular weight marker VI run in parallel. The concentration of each sample was determined by comparing the amplicon band intensity of each to the fragment of corresponding size in the Molecular weight marker VI (Appendix A4).

2.2.6 Sequencing

The ABI PRISM® BigDye™ terminator V3.1 cycle sequencing Kit with AmpliTaq® DNA Polymerase FS (Applied Biosystems, Inc. Foster City, CA) and an automated ABI prism 3100 genetic analyzer (Applied Biosystems, Inc.) was used for the sequencing reactions. Three overlapping fragments spanning full length *gag* were sequenced with gene fragment-specific primers in both the forward and reverse directions using the di-deoxy chain termination method (Sanger *et al.*, 1977). The standard di-deoxy chain termination method makes use of 2', 3' ddNTPs, which upon incorporation into the growing DNA strand by polymerases, results in chain termination. A ddNTP lacks a 3'-hydroxyl residue that is needed for phosphodiester bond formation between successive dNTPs. These ddNTPs are added to the reactions together with an abundance of dNTPs, resulting in competitive chain-extension and termination. Oligonucleotide chains are produced with varying length, differing by single nucleotides. These are then fluorescently labelled, allowing for detection upon electrophoresis.

A 15-20ng amount of purified amplicon DNA was added into PCR tubes. There were two tubes for each of the three *gag* fragments (A, B and C), one for the forward sequencing reaction and the other for the reverse sequencing reaction. One µl of 3.2 pmol of the forward and reverse primers were added into the appropriate tubes. The primers were the same primers as those used for the PCR reactions. The reaction volume was adjusted to 12µl with diethypyrocarbonate (DEPC)-treated water, which inactivates RNases. Four µl of 2.5 X sequencing buffer supplied with the kit (Appendix B3) was added and then 4µl of sequencing enzyme mix from the BigDye™ terminator V3.1 cycle sequencing kit was added, giving a total sequencing reaction volume of 20µl. The PCR cycling conditions are described in Table 2.6.

Table 2.6 Sequencing cycling conditions

Cycling condition		Time and Temperature (^{0} C)
(denaturing)		30 seconds (96^0 C)
	1 cycle	
(denaturing)		30 seconds (96^0 C)
(annealing step)		15 minute (50^0 C)
(extension step)		4 minutes (60^0 C)
	25 cycles	
-END		-END (4^0 C)

2.2.7 Sequence analysis

The three *gag* overlapping fragments were assembled into a continuous full length *gag* nucleotide sequence using ChromasPro Version 1.34 (Technelysium Pty Ltd, USA). The sequences were aligned, using ClustalW for multiple alignments incorporated in BioEdit sequence alignment editor v7.0.5 (Tom Hall, Ibis Therapeutics; Carlsbad, CA) with 1000 bootstraps. The alignment was manually edited and translated into amino acid sequences using BioEdit. The different *gag* regions were analyzed separately after alignment for contamination. Phylogenetic and molecular evolutionary analyses were performed using *MEGA* (Molecular Evolutionary Genetics Analysis) version 3.1 (Kumar, Tamura, Nei, 2004). For quality control and subtyping phylogenetic trees, the reference panel included sequences of different global HIV strains with respect to the full-length *gag* region of HIV-1, consisting of all subtypes (A-K) as well as circulating recombinant forms (CRFs) from the Los Alamos sequence database (http://www.hiv.lanl.gov/components/hiv-db/). Two sequences of each subtype and CRF were included, while subtype C reference sequences were considered as the majority of the panel since previous data showed that the majority of viruses circulating in South Africa are subtype C viruses. The chimpanzee SIV sequence was used as an outlier in all the phylogenetic trees.

The Neighbor-Joining method with the Jukes and Cantor nucleotide substitution model (Jukes and Cantor, 1969) was used to determine the phylogenetic relatedness of the sequences. For the subtype C phylogenetic tree, subtype C *gag* sequences from different parts of the world were used as the reference sequences.

REGA version 6.4.2 (de Oliviera *et al.*, 2005) software was used for recombination analysis of the study sequences in four sequential steps. First a query (study subject's) sequence was aligned with 27 group M sequences A-D, F-H, J and K and a Neighbor-Joining phylogenetic tree was drawn. One hundred bootstrap replicates were used with 70% as the cut-off value. Secondly, the process was repeated with 28 CRF reference sequences in addition to 22 pure subtype reference sequences. In the third step, the query sequence was divided into small segments and a sliding window of 400 bp was moved along the sequence in 20 bp increments. Each segment in the query sequence and the reference alignment was then analysed for recombination using bootscanning methods, implemented in Phylogenetic Analysis Using Parsimony (PAUP). Finally, the alignments were examined to determine whether they contain sufficient phylogenetic signal for subtype determination using

likelihood mapping analysis implemented in the Treepuzzle software (Strimmer and von Haesler, 1997) and the results presented were as shown in Appendix B4, Figure B1.

Genetic distance calculations were performed using the Poisson Correction (PC) distance model (Kumar and Nei, 2000). The model assumes equality of substitution rates among sites and equal amino acid frequencies while correcting for multiple substitutions at the same sites. Amino acid distances were calculated for full-length *gag* sequences between isolate sequences and peptide reagent sequences for subtype Cs (South African Du422 and Chinese C) and subtype B. For peptide reagents based on subtypes A and D, only p17p24 p2 distances were calculated because the peptides ranged encompassed these regions only.

Shannon entropy, a measure of variability in an alignment that takes into account the possible amino acid substitutions at particular position as well as their frequencies, was calculated for aligned full-length Gag sequences from the study subjects. BioEdit (Biological sequence alignment editor) v7.0.5 was used for the entropy calculations (Tom Hall, Ibis Therapeutics; Carlsbad, CA).

2.3 RESULTS

Forty subjects were recruited, twenty from Soweto in Johannesburg and another twenty from Desmond Tutu HIV Centre in Cape Town. All the study individuals were asymptomatic and were not on antiretroviral treatment.

2.3.1 Amplification and sequencing of HIV-1 *gag*

Gag sequences were generated from three overlapping fragments of full length *gag* (A, B and C) with each fragment approximately 600bp in length (a representative example is shown in Figure 2.1 a). Following purification, DNA was quantified using agarose gel electrophoresis (a representative example of a gel is shown in Figure 2.1 b) and sequenced to generate complete *gag* sequences from all 40 subjects.

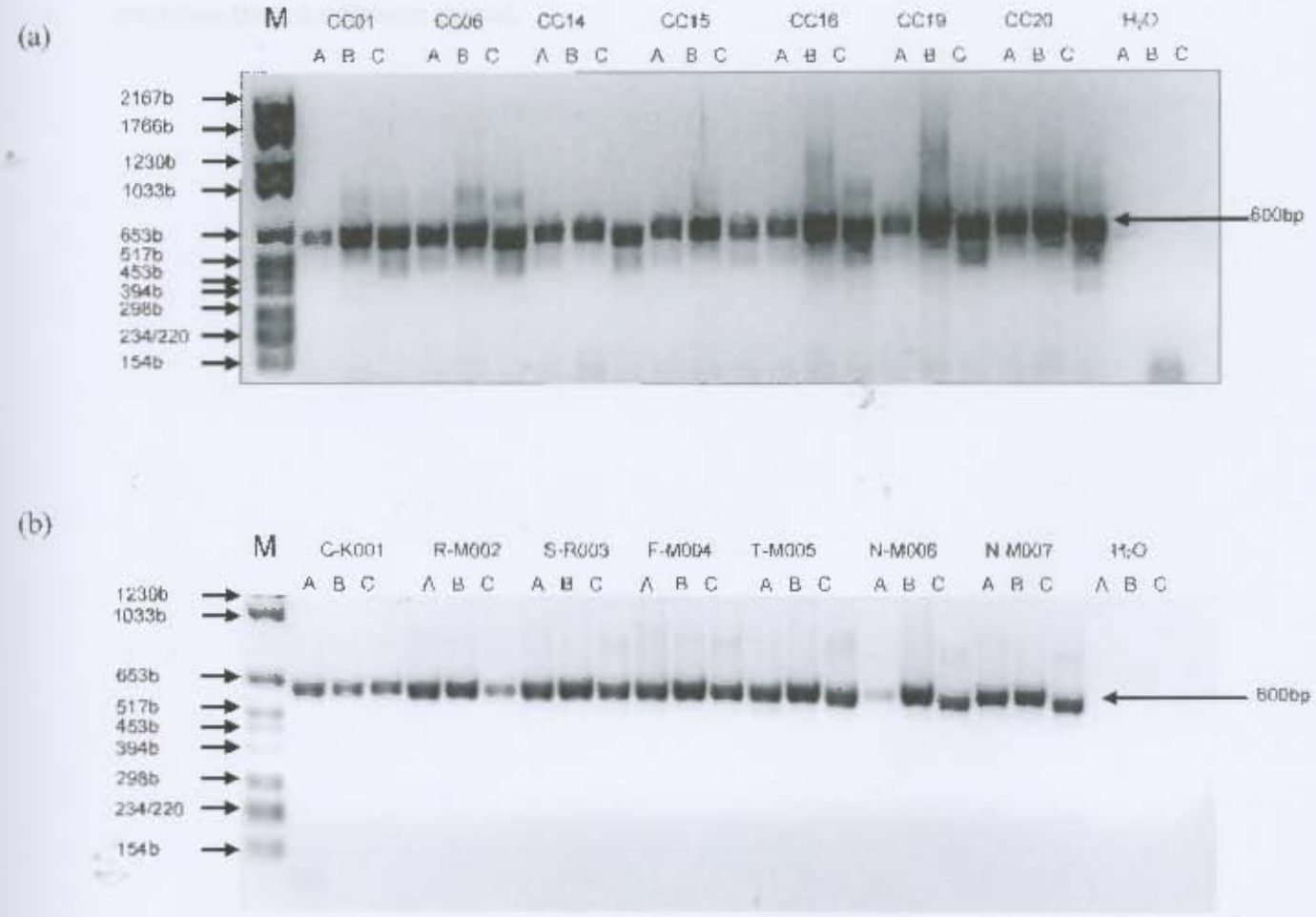


Figure 2.1 (a): Horizontal Agarose gel electrophoresis of HIV-1 *gag* DNA from second round PCR reactions. **(b)**: Agarose gel quantification of amplified HIV-1 *gag* regions after purification. The molecular weight marker VI- **M** was used (4µl in 6µl DEPC-treated water and 3µl of bromophenol blue dye).The sizes of the molecular weight marker VI bands are shown in base pairs (bp) on the left of the figure. DEPC-treated water (H_2O) was used as a PCR negative control. Three *gag* fragments of approximately 600bp sizes, A, B and C for each study sample were loaded (10µl) together with 3µl of loading dye

2.3.2 Quality control of HIV-1 *gag* nucleotide sequences

To ensure there was no PCR contamination or sample mix-ups, Neighbor-Joining phylogenetic trees were drawn for each *gag* fragment prior to sequence assembly. For the 120 fragments analyzed in three separate phylogenetic trees, there was no evidence of unusually close clustering of sequencing confirming no contamination (Figure 2.2 a-c). There was evidence that the phylogenetic relatedness was preserved across all regions in four pairs of sequences (illustrated with a red arc). Fragments from a single individual had homologous overlapping regions of 70 bp further confirming these sequences came from this individual. Sequences from two participants (T-N 010 and M-T 009) showed relatively close relatedness on all the three separate phylogenetic trees (Figure 2.2 a-c) with relatively short branch length. Two additional sets of sequences CC22, CC04 and CC17, and N-M20 and R-L12, also showed close relatedness in all the three trees. In all cases, the participants came from the same site and it is possible they are either a transmission pair, or they acquired infection from a common source.

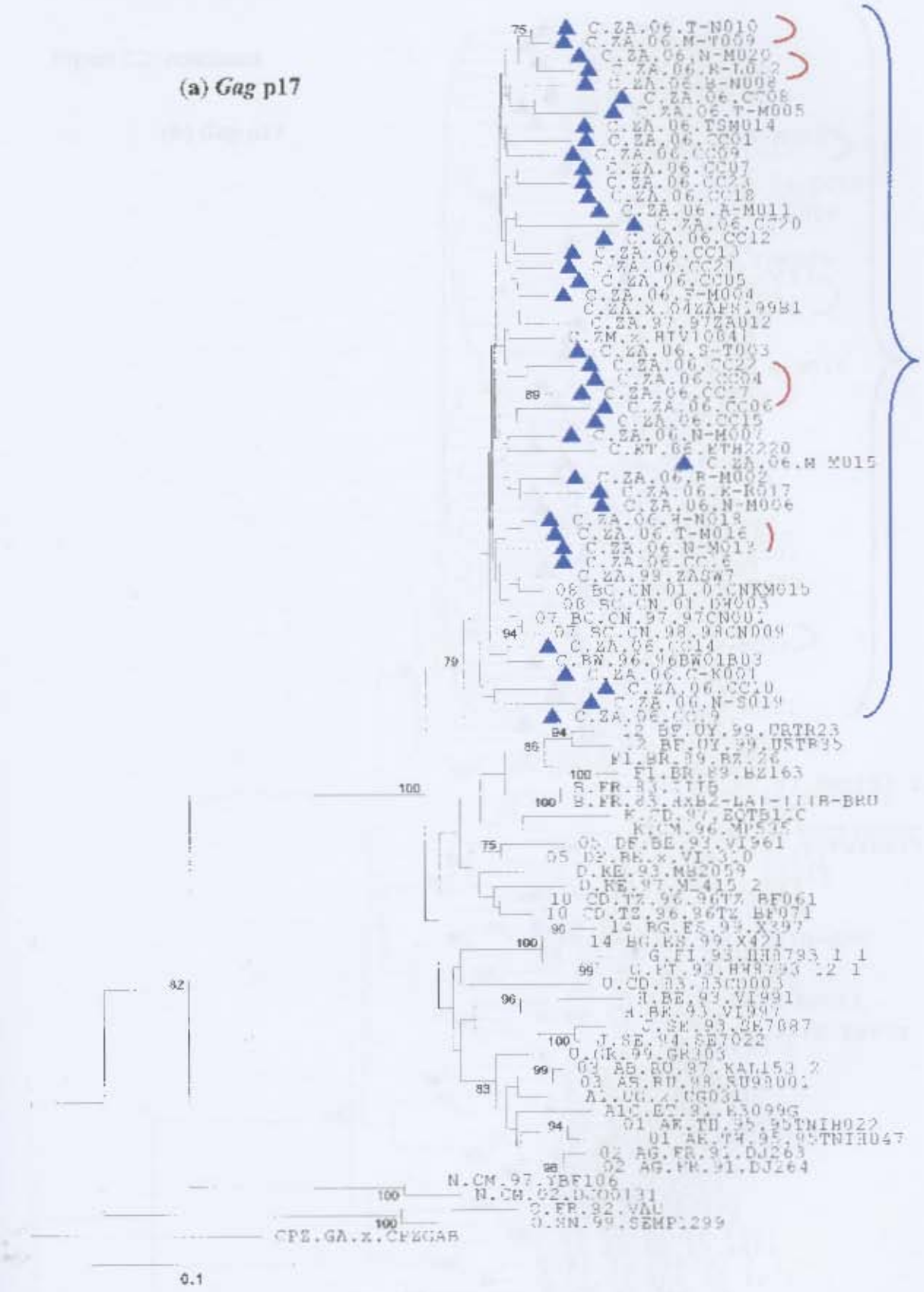


Figure 2.2 Neighbor-Joining phylogenetic trees of HIV-1 *gag* fragment sequences. (a) *Gag* p17, (b) p24 and (c) p15. The reliability of the tree was estimated from 500 bootstrap replicates and bootstrap supports above 75% are shown at the branch nodes and as well as red arcs for isolate sequences. All subtype C sequences are indicated with a right blue brace. The scale bar indicates a 10% nucleotide sequence divergence. Study sequences (N=40) described in this study are shown with blue filled triangles. Reference sequences from subtypes A-K as well as circulating recombinant forms have been included HIV groups O an N are included. CPZ.GA.x.CPZGAB, a chimpanzee SIV *gag* sequence was used as an out group.

Figure 2.2: continued

(b) *Gag* p24

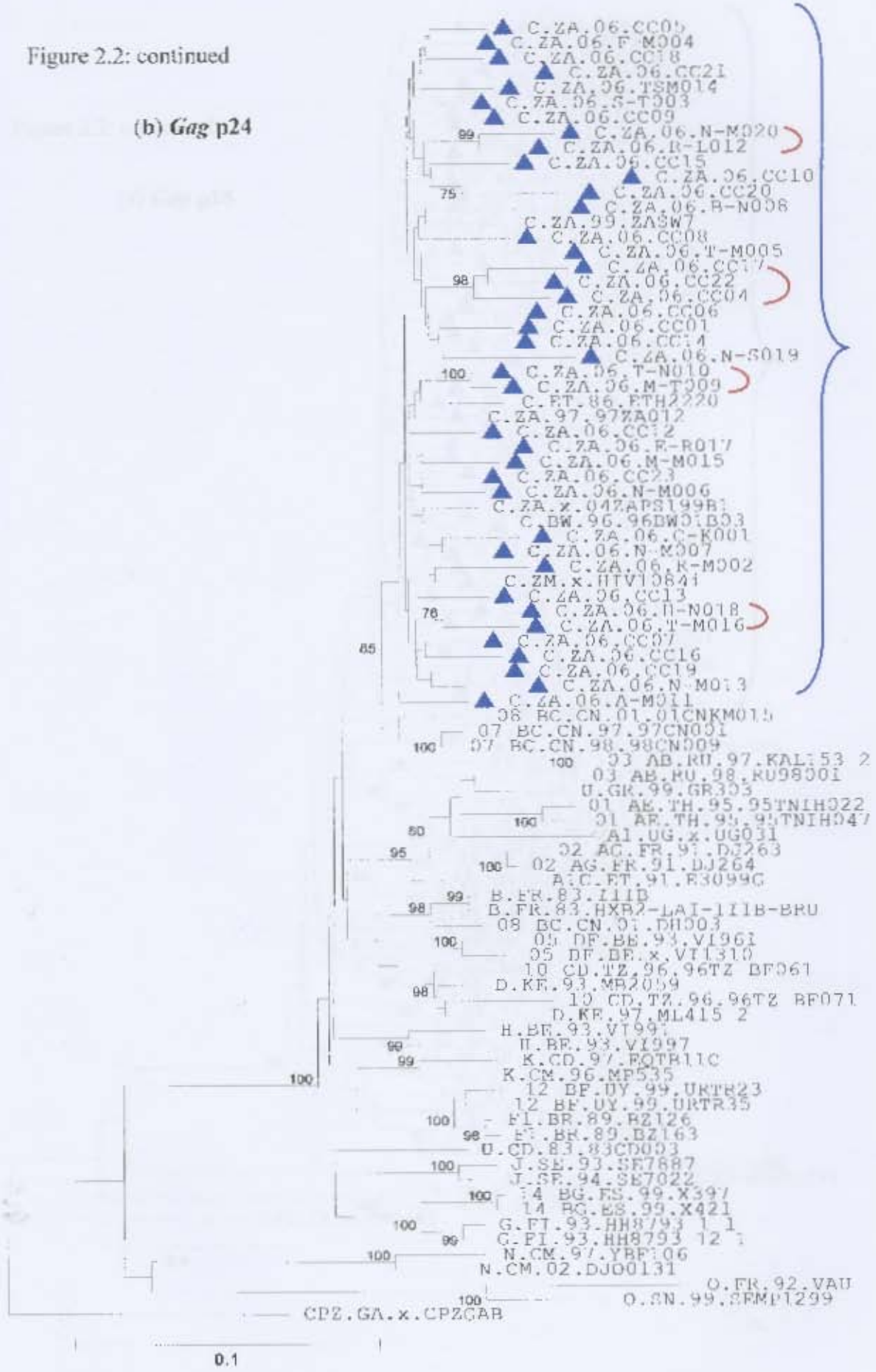
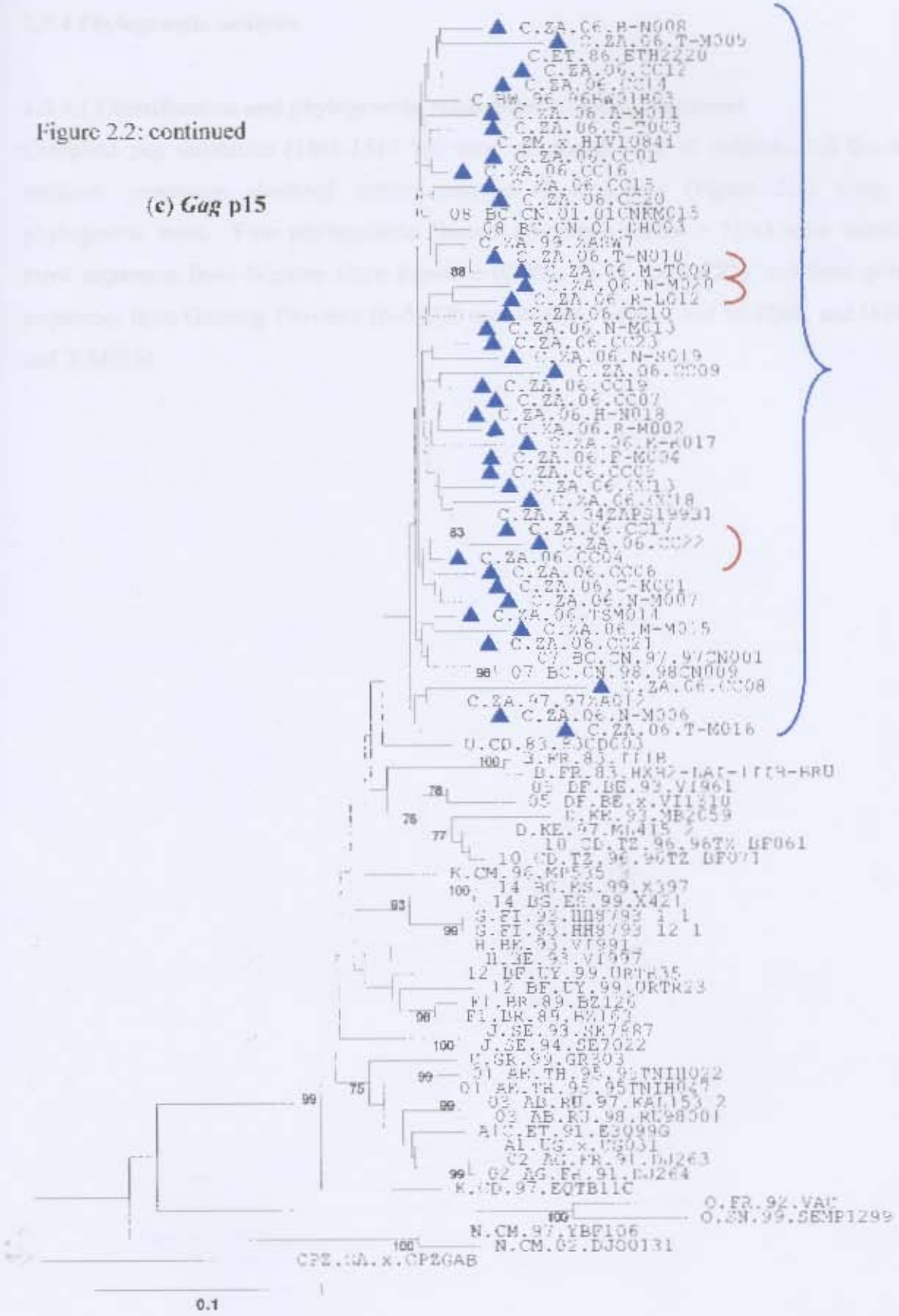


Figure 2.2: continued

(c) *Gag* p15



2.3.4 Phylogenetic analysis

2.3.4.1 Classification and phylogenetic relatedness of study sequences

Complete *gag* sequences (1461-1515 bp) were generated from 40 subjects. All the study subjects' sequences clustered within subtype C sequences (Figure 2.3) using N-J phylogenetic trees. Four phylogenetic clusters (bootstrap values > 75%) were identified, three sequences from Western Cape Province (CC04, CC17 and CC22), and three pairs of sequences from Gauteng Province (N-M020 and RL012, T-N010 and M-T009, and H-N018 and T-M016).

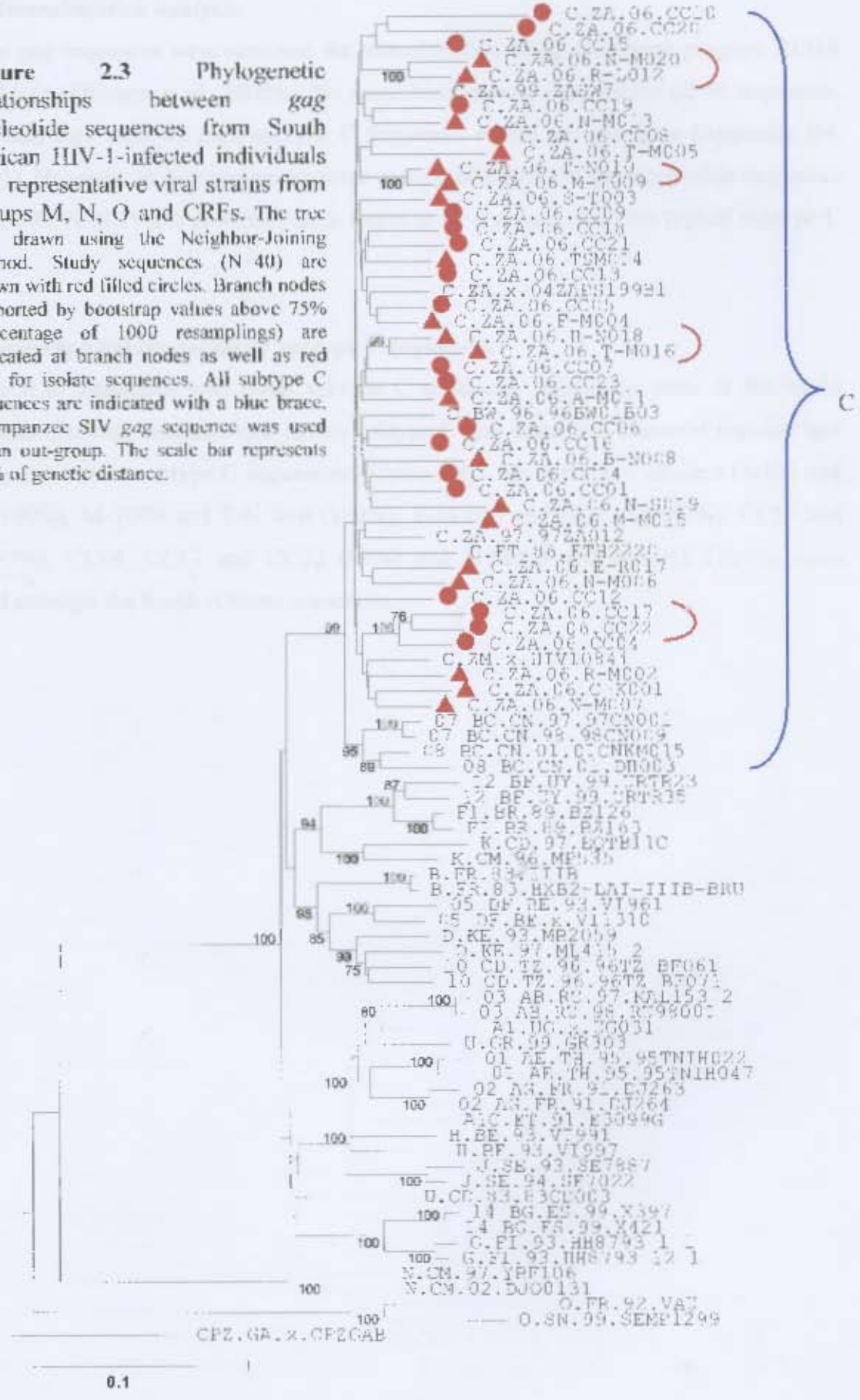


Figure 2.3 Phylogenetic relationships between *gag* nucleotide sequences from South African HIV-1-infected individuals and representative viral strains from groups M, N, O and CRFs. The tree was drawn using the Neighbor-Joining method. Study sequences (N 40) are shown with red filled circles. Branch nodes supported by bootstrap values above 75% (percentage of 1000 resamplings) are indicated at branch nodes as well as red arcs for isolate sequences. All subtype C sequences are indicated with a blue brace. Chimpanzee SIV *gag* sequence was used as an out-group. The scale bar represents 10% of genetic distance.

2.3.4.2 Recombination analysis

Complete *gag* sequences were screened for recombination using a software program REGA version 2.0 (de Oliviera *et al.*, 2005). No recombination was detected for all 40 sequences, showing that they were all pure subtype C sequences within the *gag* gene (Appendix B4, Figure B1). However, in four sequences some regions of the HIV-1 *gag* nucleotide sequences (CC01, CC04, N-S019 and R-M002) were found to be divergent from the typical subtype C sequence.

2.3.4.3 Geographical clustering of subtype C sequences

Comparison of sequences with other subtype C sequences from other parts of the world showed that Chinese, Brazilian and Indian subtype C *gag* sequences clustered together and separately from other subtype C sequences (Figure 2.4). Six subtype C clusters Du151 and Du422 (100%), M-T009 and T-N 010 (100%), T-M 016 and H-N 018 (97%), CC15 and CC20 (97%), CC04, CC17 and CC22 (99%) and N-M020 and R0L012 (100%) were identified amongst the South African sequences.

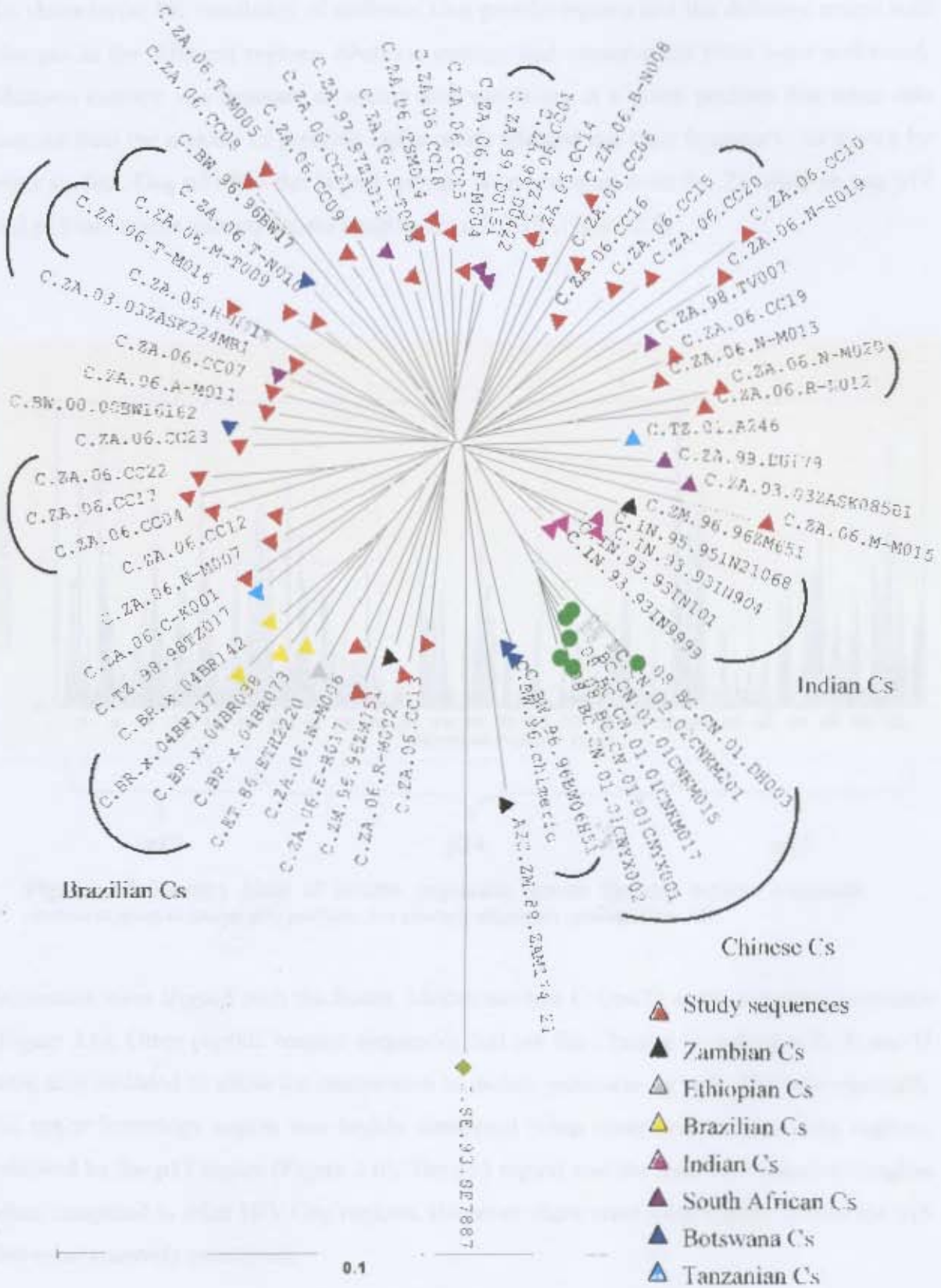


Figure 2.4 Neighbor-Joining phylogenetic relationship of HIV-1 subtype C full length gag sequences from different regions of the world. Study subjects' sequences (N=40) are shown with red filled triangles. Bootstrap values above 75% are shown with black arcs. Size of scale bar is equal to 10% DNA distance.

2.3.5 Characterization of differential conservation of HIV-1 *gag* regions

To characterize the variability of different Gag protein regions and the different amino acid changes in the different regions, Shannon entropy and conservation plots were performed. Shannon entropy is a measure of amino acid variability at a given position that takes into account both the number of possible amino acids allowed and their frequency. As shown by other studies, Gag p24 had the lowest entropy score, ranging from 0-1.25 while as gag p17 and p15 had higher entropy scores ranging from 0-2.15 (Figure 2.5).

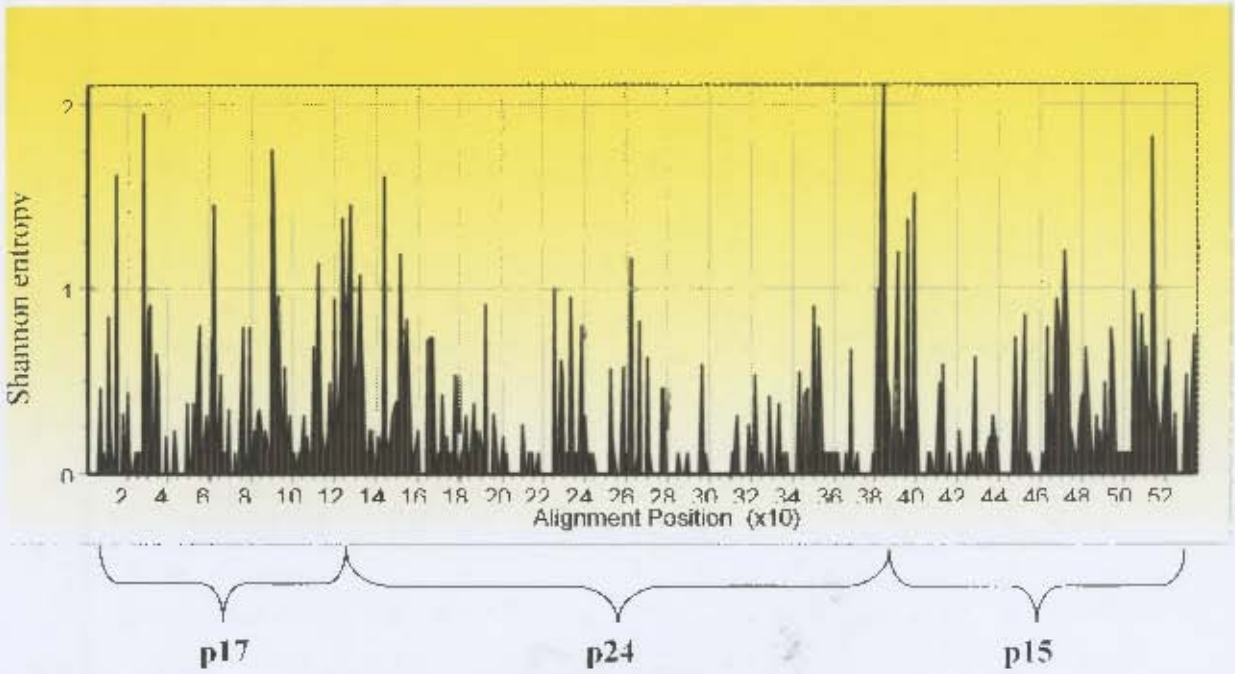
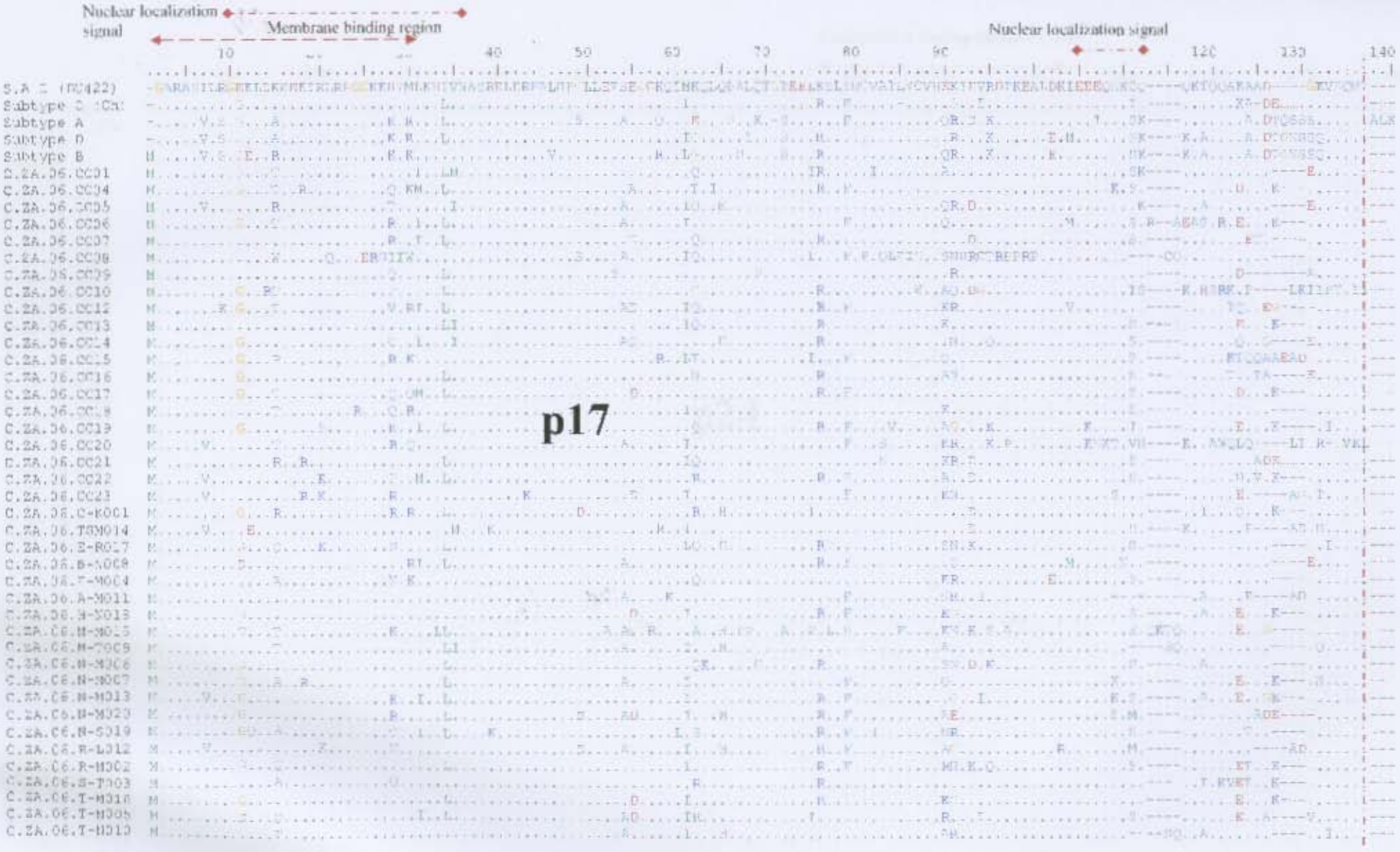
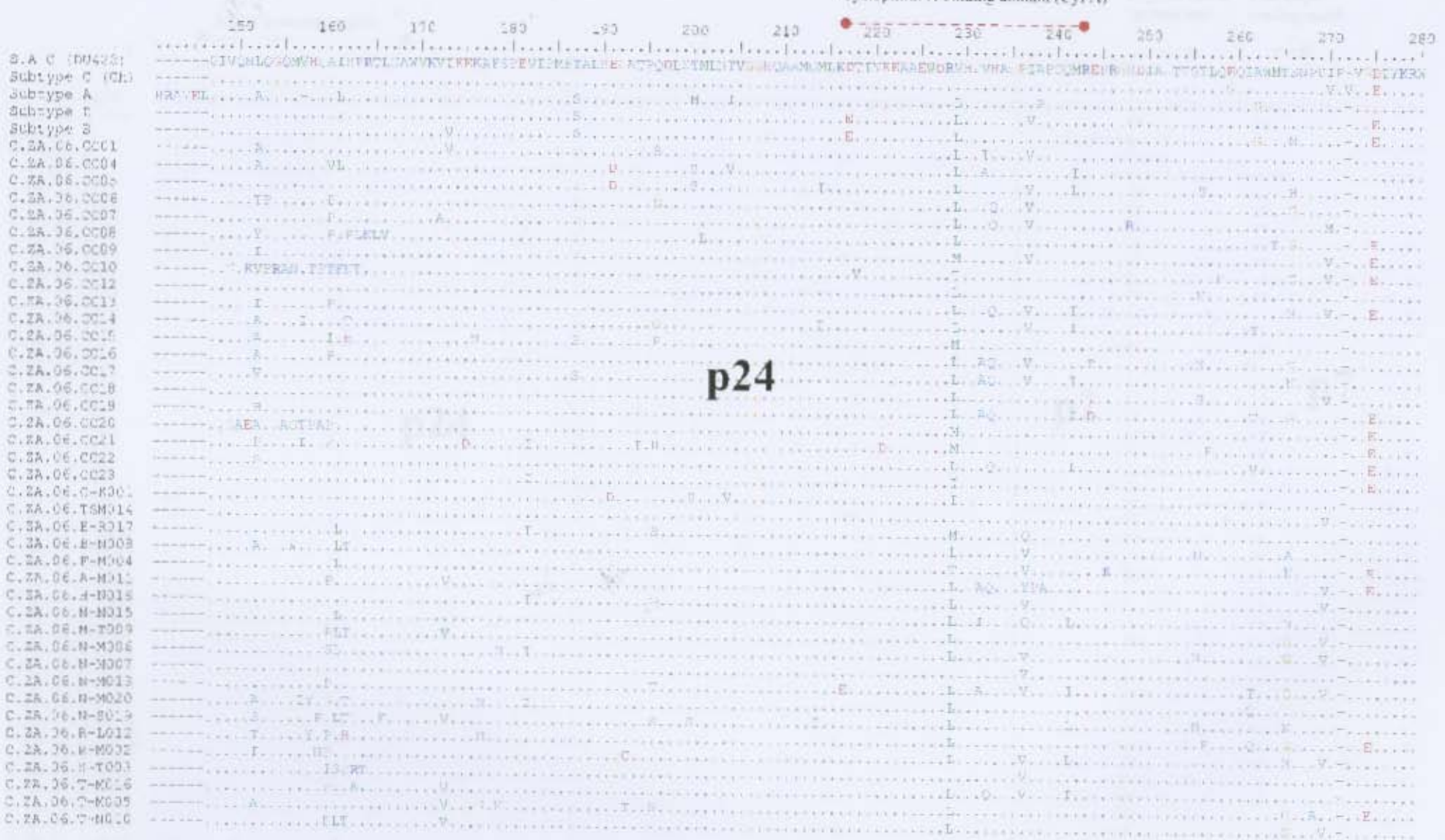


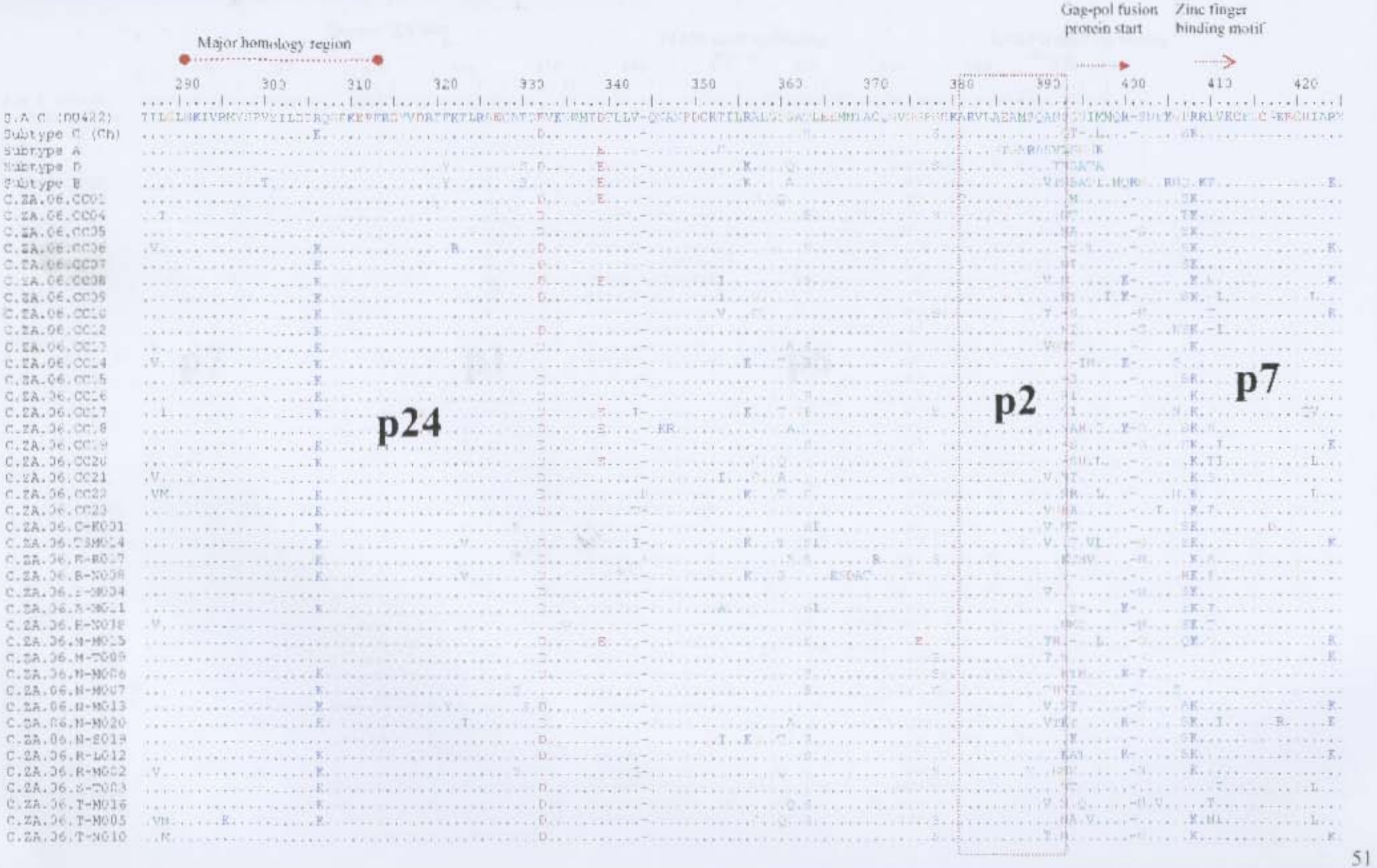
Figure 2.5 Entropy plots of isolate sequences across the gag region. Alignment position is given as amino acid position. For example alignment position 12 is 120.

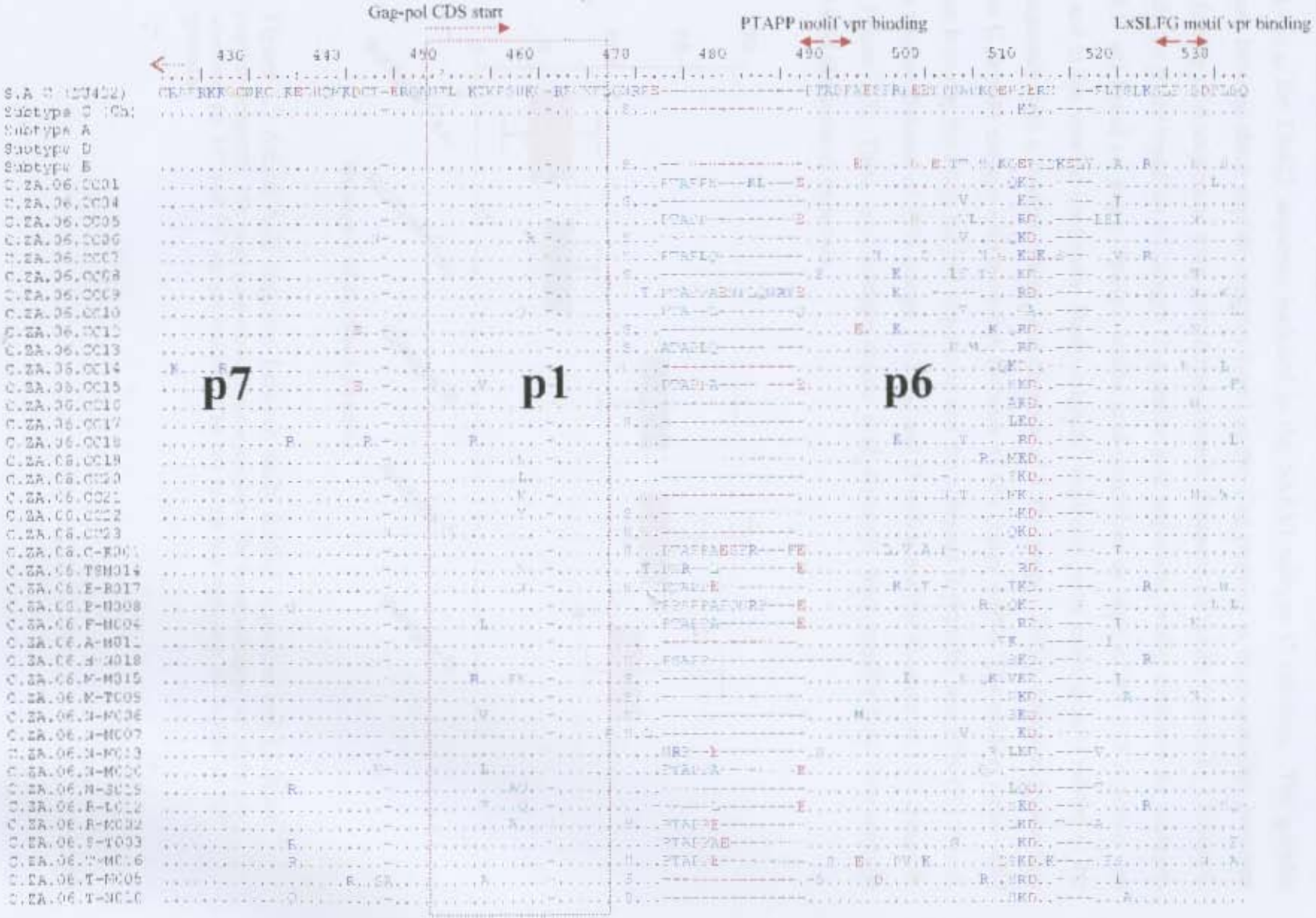
Sequences were aligned with the South African subtype C Du422 as the reference sequence (Figure 2.6). Other peptide reagent sequences that are the Chinese C, subtypes B, A and D were also included to allow for comparison to isolate sequences as well. The p24 especially the major homology region was highly conserved when compared to other Gag regions, followed by the p17 region (Figure 2.6). The p15 region was the least well conserved region when compared to other HIV Gag regions. However, there were some regions within the p15 that were relatively conserved.

Figure 2.6 The predicted alignment of HIV-1 gag amino acid sequences from South African HIV-1 strains analyzed in this study. The multiple aligned sequences where compared to ELISpot peptide reagent sequences based on vaccine candidates (South African subtype C (Du422), subtype B, partial Gag for subtypes A and D). Dots indicate amino acid identities, while the dashes correspond to gaps introduced to maintain the correct alignment. The different Gag domains are indicated.


Nuclear localization signal
Membrane binding region
Nuclear localization signal
p17


Cyclophilin A binding domain (CyPA)
p24


Major homology region
Gag-pol fusion protein start
Zinc finger binding motif
290
300
310
320
330
340
350
360
370
380
390
400
410
420
p24
p2
p7


Gag-pol CDS start
PTAPP motif vpr binding
LxSLFG motif vpr binding
430
440
450
460
470
480
490
500
510
520
530
p7
p1
p6

2.3.6 Genetic distance relationship among the major HIV-1 subtypes

In chapter three, we report on responses to HIV-1 Gag peptide sequences based on potential and existing vaccine constructs which are subtype A (HIVA vaccine) (Mwau *et al.*, 2004), subtypes B CAM-1 strain, D and two subtype Cs, one from China (C_{CH}) and another one from South Africa ($C_{S.A}$, the Du422 sequence included in the SAAVI subtype C vaccine). The genetic distances between these peptide reagents based on the Gag proteins in these candidate vaccines and infecting viral sequences were determined. Genetic distances for South African C, Chinese C and subtype B are based on full length Gag amino acid sequences, whilst distances for subtypes A and D are based on gag p17p24p2 amino acid sequences as most of the p15 region is not contained in the vaccine constructs. Study subjects' sequences were more closely related to the $C_{S.A}$ sequence with a median amino acid distance of 5.5% (range 2-9%), followed by the C_{CH} subtype C peptide reagent with a median distance of 6% (range 4-9%). The median amino acid distance between the study subject sequences and the subtypes B peptides was 12% (9-19%), A with a median distance of 13% (range 11-20%) and D with a median distance of 11% (range 8-18%, Figure 2.7). Thus, intra-subtype amino acid distances were smaller than inter-subtype amino acid distances as expected.

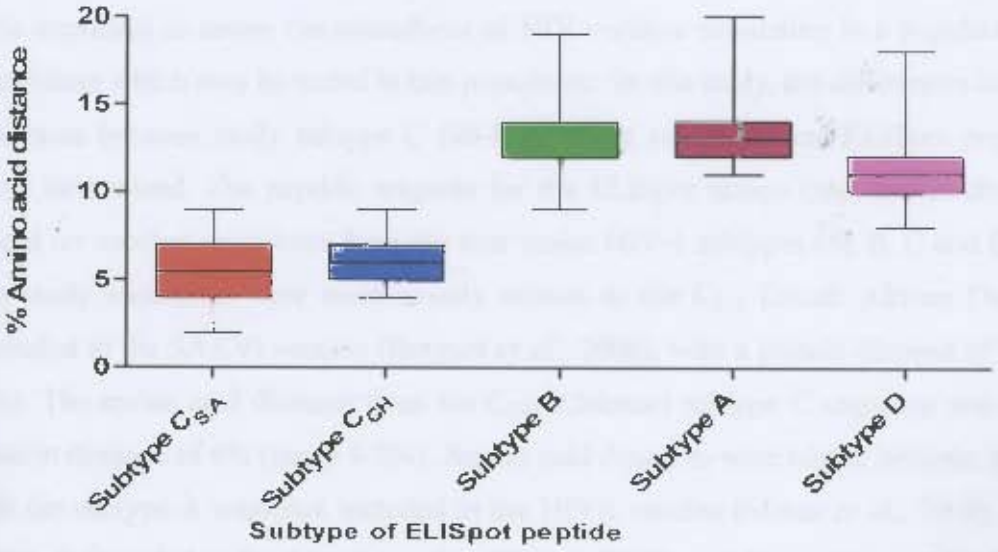


Figure 2.7 Amino Acid distances between study subject sequences and ELISpot peptide reagents. The range is represented by the whiskers. The lower and upper part of the box represents the 25th and 75th percent quartiles. The line in the box represents the median or 50th percentile.

2.4. DISCUSSION

In this study, the entire *gag* gene sequences and their predicted protein translations from 40 HIV-1 strains collected from two different provinces of South Africa, namely Western Cape and Gauteng, were genetically characterized. Phylogenetic analysis of the full length *gag* gene classified all the sequences as pure HIV-1 subtype C sequences. This classification of the sequences as subtype C viruses further support results from previous findings that the predominant circulating HIV-1 viruses in South Africa are subtype C viruses (van Harmelen *et al*., 1997; van Harmelen *et al*., 1999).

Phylogenetic analyses of HIV-1 *gag* sequences provide clear evidence of the geographical clustering of subtype C viruses from India, Brazil and China. South African sequences were distinct from Indian, Brazilian and Chinese sequences which formed monophyletic groups. This is as a result of founder effects in these regions as the epidemics started later. There is a diverse epidemic in South Africa, shown by lack of formation of monophyletic groups. This shows multiple introductions of subtype C viruses in these countries.

It is important to assess the relatedness of HIV variants circulating in a population and vaccine candidates which may be tested in that population. In this study, the differences in the amino acid distances between study subtype C full-length Gag sequences and ELISpot peptide sequences were determined. The peptide reagents for the ELISpot assays (reported in chapter three) are based on vaccine candidates from the four major HIV-1 subtypes (A, B, C and D). On average, the study sequences were most closely related to the C_{SA} (South African Du422) sequence included in the SAAVI vaccine (Burgers *et al*., 2006), with a protein distance of 5.5% (range 2-9%). The amino acid distance from the C_{CH} (Chinese) subtype C sequence was similar, with a protein distance of 6% (range 4-9%). Amino acid distances were higher between study sequences and the subtype A sequence included in the HIVA vaccine (Mwau *et al*., 2004), 12% (range 9-11%), B included in the Merck vaccine (Shiver, 2003), and D sequences, 13% (range 11-20%) and 11% (8-18%) respectively. Chapter three will investigate whether these differences are relevant with respect to cross-clade responses elicited in South African subtype C infected individuals.

Chapter 2: Genetic characterization of HIV-1 gag sequences

The sequences in the study represent a discrete region (*gag*), which encompasses approximately 15% of the HIV viral genome. The segment length has relatively conserved regions mainly within p24. The p17 region was more variable, with the p15 being the most variable region of HIV *gag* sequences. The p15 region is made up of various protein subunits namely p1, p2, p7 and p6. Within p15, two highly conserved regions occur in the p6 domain. These regions contain the Vpr binding motifs. Previous studies have shown cross-clade responses are more commonly detected in epitopes located in conserved regions (Yu *et al.*, 2005). Differences in diversity across the Gag region will enable this aspect to be explored further with respect to cross-clade responses in subtype C infected individuals.

These data on full-length *gag* gene sequences of HIV-1 subtype C strains from the Western Cape and Gauteng provinces of South Africa confirm the dominance of HIV-1 subtype C viruses in South Africa. The differences in diversity across *gag* will enable us to differentiate the impact of diversity of different proteins encoded by the *gag* gene on cross-reactive immune responses. The data generated from this study provides the basis for the investigation of cross-clade immunity, which is important in ascertaining the importance of diversity in vaccine development strategies.

CHAPTER 3

Intra- and cross-clade T cell immune responses to Gag

3.1 INTRODUCTION

3.1.1 Intra- and inter-clade HIV-specific T cell responses

The development of an antibody-based preventive HIV vaccine has been hampered by antigen-specific responses that have been strain-specific and generally unable to neutralize primary isolates (Coeffier *et al.*, 1997; Li *et al.*, 2005). Therefore, there has been increased interest in the induction of HIV-specific T cell responses in current vaccine approaches. However, one of the major obstacles in HIV vaccine development is the enormous genetic diversity of the virus, which also poses a problem in determining which immunogens to include in a vaccine.

Among all HIV proteins, Pol is the most conserved (>90%) across all HIV-1 subtypes and is approximately 850 amino acids long. HIV Nef has a conserved central region and a variable outer region. Overall, Nef is also relatively well conserved (80%) across subtypes A, B and C. Gag contains both conserved and variable regions and overall, it is relatively well conserved as well (85%) across subtypes A, B and C. In contrast, Env is less well-conserved (<80%) even within a single subtype (http://hiv-web.lanl.gov). HIV Gag forms part of many current HIV vaccine candidates. It is included in a South African AIDS Vaccine Initiative (SAAVI) DNA vaccine (Burgers *et al.*, 2006). The *gag* sequence included in this vaccine was selected based on near consensus subtype C sequence, which is the Du422 sequence (Williamson *et al.*, 2003). HIV Gag also forms part of the trivalent mixture of Adenovirus serotype 5 vectors each encoding subtype B based Gag, Pol and Nef (Shiver and Emini, 2004). HIVA, a DNA- and modified virus Ankara (MVA) - vectored candidate HIV vaccine also express the p24/p17 Gag coupled to $CD8^+$ T cell epitopes (Hanke *et al.*, 2002). The level of sequence conservation for this protein coupled with its relatively large size (approximately 500 amino acids) suggests the existence of multiple T cell epitopes that are common to diverse viruses. Studies have found that HIV-infected individuals mount robust T cell responses to HIV Gag protein (Addo *et al.*, 2003; Masemola *et al.*, 2004; Kiepiela *et al.*, 2004). Therefore, it is important to test for cross-reactive immune responses among HIV variants to the Gag protein based on current and other potential HIV vaccine candidates.

High frequencies of cross-reactive HIV-specific T cells have been detected by early studies (Cao *et al.*, 2000; Ferrari *et al.*, 1997, Fukada *et al.*, 2002). More recent studies have shown substantial cross-recognition of the major HIV-1 subtypes but with preferential targeting of the infecting subtype (McKinnon *et al.*, 2005). Furthermore, these responses were shown to be substantial for the Gag protein particularly those regions with lower entropy and higher interclade homology (Coplan *et al.*, 2005; Gupta *et al.*, 2006; Yu *et al.*, 2005). Generating further information on cross-clade HIV-specific T cell responses is important in HIV vaccine development strategies.

South Africa is faced with an enormous HIV/AIDS epidemic and a successful vaccine is urgently needed to prevent new infections. It is understood that International AIDS Vaccine Initiative (IAVI) and the South African AIDS Vaccine Initiative (SAAVI) would like to implement phase III vaccine trials in South Africa with vaccine candidates that have been tested in phase I/II trials within and outside of South Africa. Most notably, the Merck vaccine containing subtype B CAM-1 strain *gag* currently in phase II trials in South Africa, is a possible candidate for further testing in South Africa, as well as clade C candidates from China. It is thus important to generate data that explores intra- and inter-clade T cell activity in subtype C-infected individuals in South Africa with clade A, B, C and D reagents that correspond to existing vaccine constructs. Also linked with cross-clade recognition is the influence of diverse HLA backgrounds, and it is important to measure the degree of cross-reactive T cell responses in a South African genetic background. The data can then be used to assess the degree of cross-clade reactivity in a population that represents future cohorts for Phase III vaccine trials.

3.1.2 Aim of the chapter

The aim of this chapter was to investigate intra- and inter-clade Gag-specific T cell responses in HIV-1 subtype C-infected individuals from South Africa. The subtypes A, B, $C_{S.A}$, C_{CH} and D Gag peptides used to investigate immune responses are based on HIV isolate sequences included in current or planned HIV vaccine candidates.

3.1.3 Specific objectives

The specific objectives of the work were

i. To screen for intra-clade HIV-specific T cell responses using peptide pools in an ELISpot assay in 40 HIV-1 subtype C-infected individuals, using Gag peptides based on vaccine constructs from a South African subtype C isolate (strain Du422), $C_{S.A}$, and a Chinese subtype C isolate, C_{CH}.
ii. To determine cross-clade HIV-specific T cell responses using Gag peptides based on subtypes A (included in the HIVA vaccine), B (the CAM-1 strain included in the Merck vaccine) and D.
iii. To investigate the relationship between immune responses detected and infecting viral sequence obtained in chapter 2.

3.2 MATERIALS AND METHODS

3.2.1 Peripheral Blood Mononuclear Cells (PBMCs) isolation

During PBMC isolation, heparinized whole blood is layered on top of a density gradient material (Ficoll/Hypaque) and subjected to a centrifugal force. The centrifugation process results in the blood tube contents dividing into four distinct layers, first the red cells, granulocytes and the dense solution at the bottom of the tube, second the separating disc separating RBC from PBMC, third the Ficoll layer containing PBMCs and lastly plasma layer. He top plasma layer is pipetted and the PBMC layer carefully transferred to a tube for further processing.

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly anticoagulated blood from 40 study participants in acid citrate dextrose (ACD) vacutainers, using standard Ficoll-Hypaque (Sigma-Aldrich, UK) density gradient centrifugation. Leucosep separation tubes (Greiner bio-one, USA) were used. The Ficoll was allowed to reach room temperature and 15ml was pipetted into 50ml Leucosep tubes. This was centrifuged for one minute at 1000×*g* (2300rpm in a Heraeus 1.0R centrifuge). The blood in the vacutainers was mixed and 30ml poured onto the separating disc. This was centrifuged for 15 minutes at 1000×*g* as before. The top layer of plasma was removed without disturbing the PBMC layer. Plasma was stored at -80^{0}C in cryovials. The PBMC layer was carefully transferred to a 50ml falcon tube, diluted to 50ml using PBS 1% FCS (Invitrogen, USA) and centrifuged at 250×*g* for 10 minutes. The supernatant was discarded and the pellet resuspended in 5ml Phosphate Buffered Saline (PBS) 1% Fetal Calf Serum (FCS). PBMCs were counted and stored at 10-20 x 10^6 per cryovial in 90% FCS 10% dimethyl sulphoxide (DMSO) and stored in liquid nitrogen.

3.2.2 Cell counting

Two methods were employed for cell counting. Manual cell counting was performed for the ELISpot screening assays whilst the automated Guava cell counting procedure was performed for the confirmatory ELISpot assays.

3.2.2.1 Trypan blue counting

The Trypan blue dye exclusion cell counting method is based on the principle that live cells have an intact cell membrane that excludes certain dyes such as Trypan blue, whereas dead cells do not. A cell suspension is simply mixed with a dye and visually examined to determine whether cells take up or exclude the dye (Strober, 1997). Cell suspension (15µl) for each sample was mixed with an equal volume (15µl) of Trypan blue (Sigma, USA) exclusion dye. The mixture was mounted onto a haemocytometer and visually examined and counted under a microscope (Olympus CX21FS1, Olympus, China) to determine the numbers of live and dead mononuclear cells. The chamber has a volume of 10^{-4} cm^3 and total cell number per ml was obtained by assuming that $1cm^3$ was equivalent to 1ml and then multiplying the average of the top left and lower right quadrant cell numbers 10^4.

3.2.2.2 Guava cell counting

The assay principle is based on the different permeability of DNA-binding dyes that are fluorescently labelled. A first fluorochrome enters all cells and binds to DNA and fluoresces at its characteristic wavelength. A second fluorochrome enters only dead cells, binds to DNA and fluoresces at a different wavelength. Fluorescence intensities are then used to calculate the numbers of dead and live cells. A bead sample was prepared by diluting 20µl of beads in 380µl of diluent solution and acquired on the Guava PCA using the CytoSoft™ software in order to adjust the machine's settings. A 1:20 dilution of cells was prepared by diluting 10µl of cells in 190µl of GuavaVia Count Solution and left in the dark for 8 minutes. Samples were acquired on the Guava PCA using the CytoSoft™ software and total cell numbers and their viability determined (Guava Technologies, Inc, USA).

3.2.3 ELISpot assay

The ELISpot assay is a simple and highly sensitive assay for the analysis of cytokine production at the single-cell level. It is particularly useful for analyzing specific immune responses to whole antigens or peptides. The assay is performed in 96-well microtiter polyvinyledene plates. In the first step, the wells are coated with high affinity monoclonal antibody to the cytokine to be investigated. Cells are added

(usually 100 000/well) and incubated for 18-24 hours in the presence of antigen. During this period antigen-specific responding cells release the cytokine, which is captured in the immediate vicinity of the cells. Cells are removed by washing and a biotinylated antibody directed to a second epitope on the cytokine is added. Streptavidin conjugated with HRP (horseradish peroxidase) is added. Finally, a precipitating substrate for HRP is added and the plates are incubated until spots emerge at the site of the responding cells (a period of several minutes). The spots are examined and counted using an ELISpot plate image analyzer with spot counting software. Calculating the number of spots according to the number of cells added to each well gives the frequency of the responding cells.

3.2.3.1 Peptides

The peptide sets used in the cross-clade study belong to Gag subtypes A, D, consensus B, $C_{S.A}$ (strain Du422) and C_{CH} (from China). Subtype B peptides were provided by the National Institute of Health AIDS Research and Reagent Repository, while subtypes $C_{S.A}$, C_{CH}, A and D were provided by the International AIDS Vaccine Initiative. The two subtype Cs and subtype B peptides spanned the full length Gag protein while subtypes A and D covered the p17, p24 and p2 regions. A pool and matrix approach was used in which five pools were made up for each of the five peptide variants (Appendix C, Table C1) and 24 matrix pools were designed to include all the single Gag peptides, which make up the five different peptide variants (Appendix C, Table C2). The consensus Gag B peptides were available as 1mg lyophilized peptides while the other four peptide variants were supplied at 500µg/peptide. Single peptides from the five peptide variants were reconstituted to 10µl aliquots of 10mg/ml as stocks and then further reconstituted to 30µg/ml as peptide pools or individual peptides and stored at -80^{0}C. The peptides were used at a final concentration of 1.5µg/ml in the ELISpot assay. CEF peptide pool was used as a positive control on all tested PBMCs. The CEF peptide pool constituted a panel of 32 8-11-mer CMV, EBV and Flu virus peptide epitopes recognized by $CD8^+$ T cells. The pool was reconstituted at 20µg/ml in 90% PBS/10% DMSO and stored at -80^{0}C. An ELISpot worksheet (Appendix C, Table C3) was completed with each assay performed.

3.2.3.2 Thawing of PBMCs

PBMC vials were removed from liquid nitrogen storage and placed on dry ice while waiting to be thawed. The vials were thawed rapidly in a water bath at 37^0C. The cells were immediately added to a 50ml falcon tube and 10ml of Roswell Park Memorial Institute (RPMI) with 10% Fetal Calf Serum (FCS) (R10) media was added drop wise whilst swirling the cells. R10 was added to the cells up to 25ml and they were centrifuged at 250 x *g* (relative centrifugal force-*g*) for 10 minutes. The supernatant was discarded and the pellet was resuspended in 500µl of freshly prepared deoxyribonuclease (DNAse, 0.2mg/ml) solution (prepared as 1 in 10 dilution with R10 media) and left for 2 minutes. R10 was added to the resuspended cells up to 25ml and cells were again centrifuged for 10 minutes at 250 x *g*. The supernatant was discarded and the pellet resuspended in 5ml of R20 (RPMI with 20% FCS) media. The cells were incubated at 37^0C, 5% CO_2 in an incubator (Thermo Electron Corporation, USA) with the caps slightly loosened.

3.2.3.3 Plate coating

Each well of a 96-well polyvinyledene plates (Microsep Millipore Products, France) was coated with 50µl of 5mg/ml capturing antibody, mAb 1-D1-K (MabTech, Sweden). The coated plates were gently tapped to ensure that the entire membrane surface was completely covered with the coating monoclonal antibody. The plates were sealed with self adhesive plate seal (Brand products, Denmark) and incubated overnight at 4^0C.

3.2.3.4 Cell stimulation

On the following day, the coated plates were washed three times with 200µl/well of sterile PBS in a Biosafety class II hood. Each plate was blocked with 100µl/well of complete R10 media and incubated at room temperature for at least 2 hours.

The PBMC samples were removed from the 5% CO_2 incubator and washed with 25ml of R10 and centrifuged at 250 x *g* for 10 minutes. The cell pellet was resuspended in 5ml of R10 and counted as described in section 3.2.2.1 R10 was added to the cells up to 25ml and centrifuged as before. The supernatant was discarded and the cells were resuspended at $2x10^6$ cells/ml.

The blocking R10 media was discarded from the plates without drying the plates completely. Fifty microlitres of peptide pools described in section 2.3.3.1 were added into each well at a final concentration of 1.5μg/ml. The peptide pools were added in duplicate, as well as 24 different matrix pools (Appendix C, Table C2) as shown in the plate layout in Appendix C, Table C3. Fifty microlitres of 8-11-mer CEF (BD Biosciences, USA) peptide pool (described in section 3.2.3.7) was added at a final concentration of 1.5ug/ml, and 50μl of Phytohaemagglutinin (BD Biosciences, USA), a non-specific stimulator was added last to avoid contamination. The cells were added last and were stimulated overnight for 18 hours in a humanized 5% CO_2 incubator at 37^0C.

3.2.3.5 Membrane development

The following day, the plates were removed from the incubator and washed six times with 200μl/well of PBS containing 0.05% Tween 20 (PBS-Tween) (Sigma, USA) using a plate washer (ELx50 Auto Strip Washer, Bio-Tek Instruments, USA). Excess fluid was removed by tapping on an absorbent towel. Fifty microlitres per well of biotinylated anti-human IFN-γ monoclonal antibody clone 7-B6-1 (mAb7-B6-1, MabTech Sweden), diluted to 2μg/ml in PBS-10% FCS was added. The plates were incubated for 3 hours at room temperature.

Two hundred microlitres per well of PBS 0.05% Tween was used to wash the plates six times and Streptavidin-Horse-Radish Peroxidase (HRP) (BD Pharmingen, Canada) at 1:500 with PBS-10% FCS was added. This was incubated at room temperature for an hour. The plates were washed again six times with 200μl/well of PBS-Tween. The membranes of the wells were developed with 100μl/well of Nova Red substrate (Vector Laboratories, CA) for six minutes in the dark. The reaction was stopped by emptying the wells and rinsing in cold tap water.

The number of cells producing IFN-γ was counted for each well using an ELISpot plate reader (CTL Analyzer, CTL Technologies, Cleveland, OH) and expressed as spot forming units per million (SFU/10^6) PBMC.

Peptides in pools and matrix pools that gave positive responses in the ELISpot screening assays were tested individually to determine the reactive peptide in IFN-γ ELISpot assay. These peptide confirmation assays were performed on two selected individuals chosen because of their broad predicted reactivity.

3.2.3.6. Test acceptance criteria

A response was considered positive if the SFU/10^6 PBMC exceeded 100 after background subtraction. An assay passed if there were less than 5 spots in each of the media control wells, not more than 100 spots in each of the media and cells wells and if not less than 400 spots were present in the PHA positive control wells. Details of the assay controls and test acceptance criteria are presented in Appendix C.

3.2.3.7 ELISpot controls

As progress is made towards developing a safe and effective HIV-1 vaccine, there must be an assay that is robust and sensitive and the ELISpot assay is one such assay (Mashishi and Gray, 2002). However, the reagents that can be used as positive control must be optimized and standardized. Although mitogens such as PHA provide a quantitative answer to whether the assay works, they do not test antigen-specific T cell stimulation. The use of the same antigen-specific stimulation in different assays rather than mitogen stimulation is also useful for standardizing the IFN-γ assay as well as to monitor inter- and intra- assay variability.

Antigen-specific stimulation in the current study was performed by testing study individuals for their ability to recognize peptides based on CEF, which are 8-12 amino acid long peptides arranged into 32 pools with sequences derived from Cytomegalovirus, Epstein - Barr virus and influenza virus. CEF peptides can be used for the stimulation of IFN-γ release from $CD8^+$ T cells in individuals with defined HLA types that have been exposed to the viruses. It covers 15 different HLA class 1 alleles, in particular HLA A1, A2, A0201, A3, A11, A24, A68, A6081, B7, B8, B18, B27, B35, B44, B0702 (Currier *et al.*, 2002).

The negative control wells consisted of six unstimulated PBMC and two unstimulated wells for the QC sample per plate. Each plate also had six wells containing R10

(media only). Positive control wells consisted of two PHA-stimulated PBMC and two PHA-stimulated wells for the QC sample per plate. The control wells consisted of two CEF stimulated PBMC and two CEF-stimulated QC sample on each plate.

3.2.4 Analysis of immunodominant peptides

Reactive peptides that had >1000 SFU/10^6 PBMC were classified as immunodominant peptides. These peptides were analyzed for previously defined epitopes using the Epitope Location Finder tool at the Los Alamos immunology database (http://www.hiv.lanl.gov/content/hiv-db/ELF/epitope_analyzer.html). The tool looks for probable epitopes using previously defined epitopes on the database based on the submitted HLA type.

3.2.5 Calculation of peptide variability

Shannon entropy is a measure of the amino acid variability at a given position that takes into account both the number of possible amino acids allowed and their frequency. Entropy in each amino acid position is calculated as $-\sum P_{aa} \log P_{aa}$, where P_{aa} is the proportion of each amino acid in the respective position. When only a minority of a peptide sequence had gaps in a position, the position was included and the gaps were treated as separate symbols. A Shannon entropy score was calculated for each amino acid position of 5 corresponding peptide sequences for the 32 peptides that were reactive in two individuals. An average entropy score for all positions in each of the reactive peptides was determined to provide a single value that characterizes the overall variation of each peptide.

3.2.6 Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Prism Software, San Diego, California USA, www.graphpad.com). All data were analyzed by use of non-parametric statistics. Wilcoxon-signed rank tests of the difference between medians were used for significant differences between clade-specific responses. The non-parametric Mann-Whitney (also called the rank sum) test for un-matched pairs was used for comparison of entropy scores for different peptide categories. Correlations were determined using the non-parametric Spearman's correlations. For cross-clade reactivity ratios correlation analysis, immune responses

were log transformed and the non-parametric Spearman correlation performed. All tests were two-tailed and were considered significant if the P value was < 0.05.

3.3 RESULTS

Thirty-nine HIV-1-infected individuals were screened for HIV-1-specific T cell responses using a panel of 540 peptides spanning the complete HIV-1 Gag protein from five different HIV-1 sequences, two of which were from the same subtype. The peptides in the pools and matrices that gave positive responses in the first screening ELISpot assays were confirmed using a second confirmatory ELISpot assay.

Table 3.1 Characteristics of study subjects*

Study Individual	Age (Years)	CD4 count (Cells/µl)	Study Individual	Age (Years)	CD4 count (Cells/µl)
CC01	28	360.0	**C-K001**	31	561.4
CC04	28	474.0	**R-M002**	30	909.5
CC05	32	610.0	**S-T003**	29	578.0
CC06	22	956.0	**F-M004**	34	348.9
CC07	28	355.0	**T-M005**	27	637.8
CC08	33	752.0	**N-M006**	27	1321.4
CC09	27	453.0	**N-M007**	35	1010.5
CC10	24	590.0	**B-N008**	28	1437.5
CC12	47	536.0	**M-T009**	33	605.9
CC13	38	414.0	**T-N010**	34	428.5
CC14	47	510.0	**A-M011**	46	637.0
CC15	27	452.0	**R-L012**	19	628.2
CC16	25	590.0	**N-M013**	28	573.6
CC17	40	884.0	**TSM014**	26	295.6
CC18	27	350.0	**M-M015**	23	502.0
CC19	23	569.0	**T-M016**	29	702.6
CC20	31	388.0	**E-R017**	35	999.2
CC21	46	347.0	**H-N018**	29	471.5
CC22	31	420.0	**N-S019**	34	1167.8
CC23	31	465.0	**N-M020**	19	554.3

* All study subjects were heterosexual Xhosa people. They were all ARV-naive at enrolment. The median age was 28 years (ranging 22-47 years). The median CD4 count was 492 cells/µl (range 295-1437.5 cells/µl).

Study individuals were recruited from two provinces of South Africa, namely the Gauteng and Western Cape provinces. The majority of the individuals were all Xhosa people. 15/40 individuals had CD4 counts below 500 cells/µl and only 3/40 individuals had CD4 counts above 1000 cells/µl (Table 3.1). However, all the study volunteers were not on antiretroviral (ARV) treatment and were asymptomatic.

3.3.1 Quality control of the ELISpot assay

Thirty nine of the forty individuals were tested for their response to CEF peptides as a positive control. One individual was excluded from screening for T cell responses due to a shortage of sample available. Eighty-seven percent (34/39) study individuals had positive IFN-γ responses to CEF, while the remaining 13% (4/39) (CC 13, HN 018, B-N 008, N-M 006 and E-R 017) did not have any IFN-γ responses to these peptides (Figure 3.2). The median CEF-specific T cell response was 448 SFU/10^6 PBMC (range 0-9 917 SFU/10^6 PBMC). It is possible that the individuals who did not have responses to CEF peptides were not exposed to the viruses before or they did not have the necessary restricting HLA types.

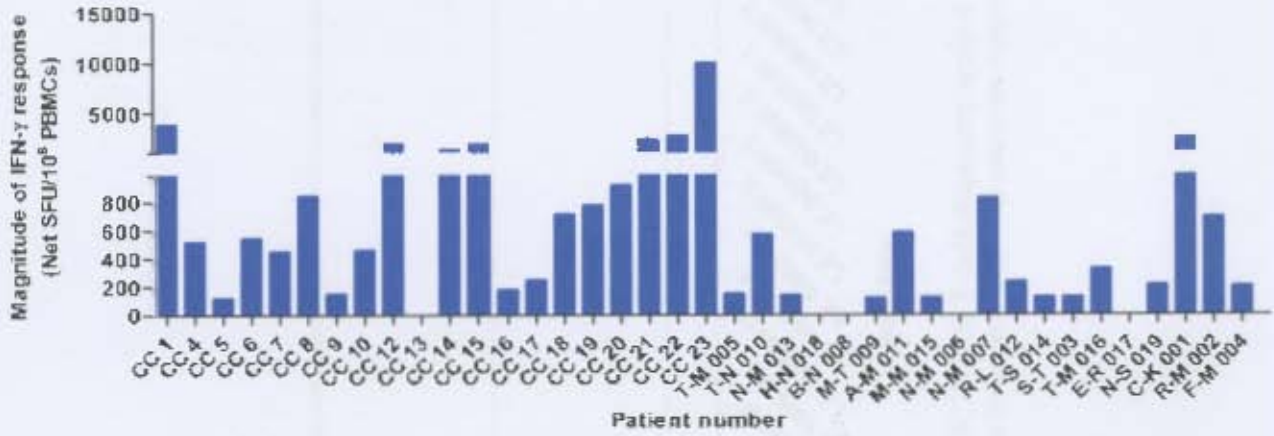


Figure 3.1 Study subjects' responses to CEF peptides. PBMCs were plated at 100 000 cells/well and the number of CEF-specific T cells producing IFN-γ were normalized to spot forming units per million (SFU/10^6) PBMC.

In each ELISpot assay, a quality control sample (NICD 063) of known reactivity to the CEF peptide pool was included, as outlined in section 3.2.3.7. In order to monitor intra- and inter-assay variability, the distribution of the quality control sample reactivity was determined and plotted in a Levy-Jennings plot (Figure 3.3). The magnitude of CEF-specific IFN-γ producing T cells expressed as SFU/10^6 PBMCs fell within the mean + 2 SD. However, assays carried out on 18th of September 2006, had magnitude of response below the mean - 2 SD. This may be due to technical reasons or detection solutions prepared incorrectly on this day, or cells counted incorrectly. Generally, there was low inter- and intra-assay variability.

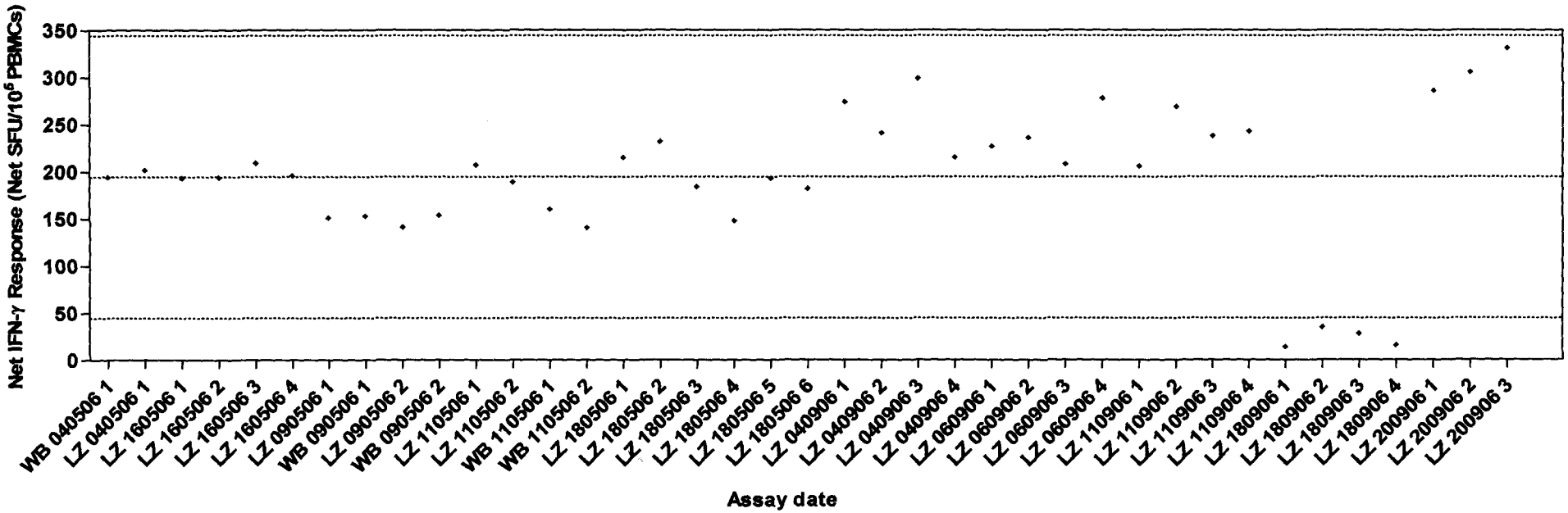


Figure 3.2 Levy-Jennings plot of quality control sample (NICD 063) T cell responses against CEF peptides. The middle dotted line represents the mean of the responses and the two lower and upper dotted lines represent the mean ± 2 SD. The assays are represented by the initials of the operator, followed by the date and the plate number for the assays performed that day.

Thirty-nine study individuals were screened for HIV-specific T cell responses against HIV Gag peptides arranged in pools and matrix pools. The total magnitudes of response were determined for all peptide sets for each study individual for both pool responses and matrix responses. The relationship between pool responses and matrix responses was determined using the non-parametric Spearman rank correlation analysis. For an ELISpot pool matrix assay to be successful, responses in pools should be accompanied by responses in a matrix containing at least one of the peptides found in the pools. This enables reactive peptides to be identified. Pool and matrix responses should therefore have a positive correlation. As expected, matrix responses correlated strongly with pool responses ($p < 0.0001$, $r = 0.9779$, Spearman rank correlation) (Figure 3.4), showing that peptides detected in the matrix were present in the pools with positive responses.

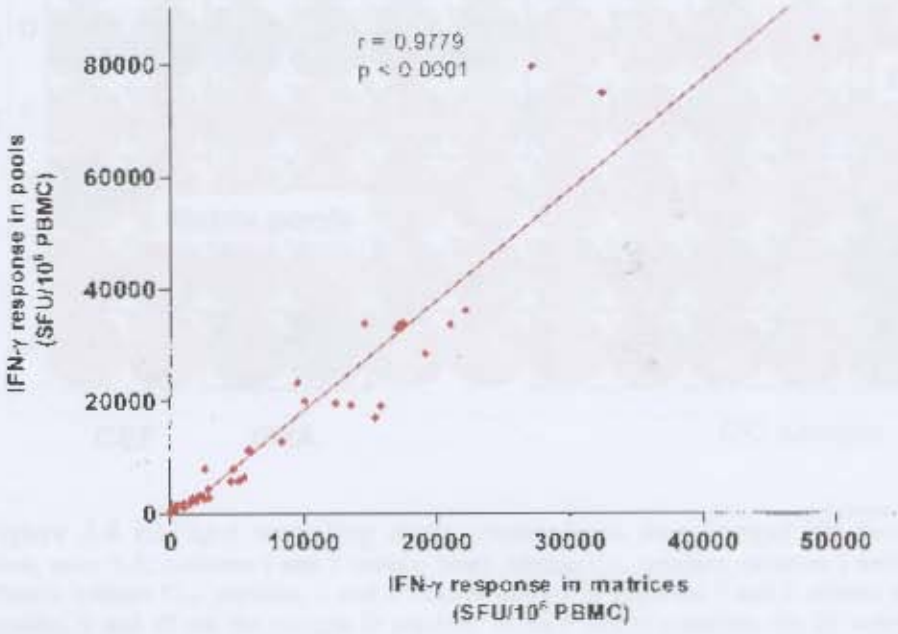


Figure 3.3 The relationship between total response in pools and total response in matrices. The magnitude of T cell responses was determined by adding magnitudes for all pools and matrices which came out positive after background subtraction for each individual. Each data point represents an individual's response to all the five HIV-1 peptide sets in pools as well as in matrices. The relationship between the responses was determined using the non-parametric Spearman correlation analysis with a 2-tailed p-value.

3.3.2 Representative of an ELISpot assay

Figure 3.5 shows a representative ELISpot assay plate for study individual CC10. The individual responded to CEF peptides and PHA, with PHA responses exceeding 400 spots per well. The media only wells (5 and 6 in row H, 11 and 12 in rows D and E) had less than 5 spots per well and the negative control wells (rows A-C, columns 11and 12) had less than 100 spots per well. The study subject responded to all HIV-1 subtypes. Remarkably, some of the peptide responses were even higher than responses to PHA in this individual.

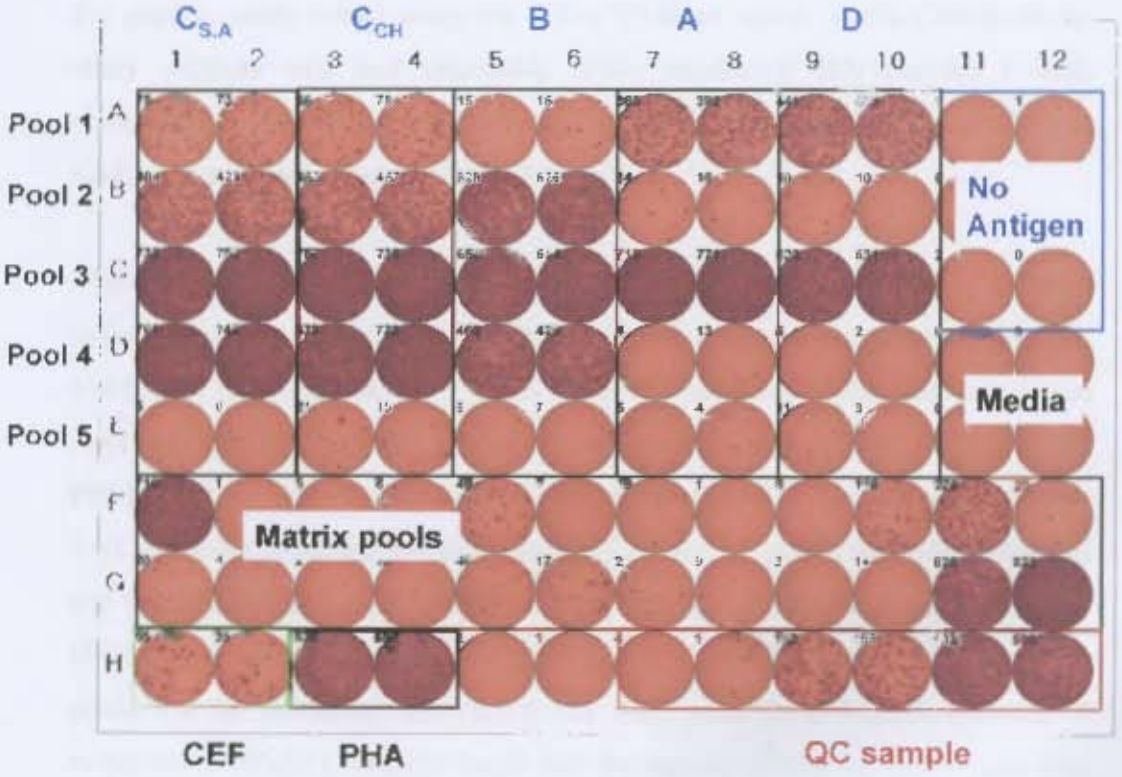


Figure 3.4 ELISpot screening assay. Peptide pools were arranged into five different pools, rows A-E: columns 1 and 2 contain South African $C_{S.A}$ peptides, columns 3 and 4 contain Chinese subtype C_{CH} peptides, 5 and 6 contain subtype B peptides, 7 and 8 contain subtype A peptides, 9 and 10 are the subtype D peptides. Rows F and G constitute the 24 matrices used. Rows A-C, columns 11and 12 contain cells and media. Row H columns 1 and 2 contain CEF peptides, columns 3 and 4 contain PHA, columns 5, 6 and 11 and 12 in rows D and E contain media alone. Row H, 7-12 contain the quality control sample with 7 and 8 containing QC and media, 9 and 10 containing QC+CEF and 11 and 12 containing QC+ PHA as shown in the figure.

3.3.3 Cell-mediated immune responses to clades A, B, C and D

3.3.3.1 Magnitude and breadth of HIV-specific T cell responses

Individuals were examined for their T cell responses to five different HIV Gag peptides sets from four different subtypes, namely two subtype Cs, subtypes B, A and D using the IFN-γ ELISpot assay. This was performed to investigate whether individuals can mount robust T cell responses to the Gag protein based on the different major HIV-1 subtypes. HIV-specific T cells were detectable in 97.4% (38/39) of the study individuals (Figure 3.6). The remaining individual (study individual A-M 011) did not have HIV-specific T cells recognizing any one of the peptide pools tested using the IFN-γ ELISpot assay. 100% (38/38) of the study subjects who had detectable IFN-γ producing HIV-specific T cells recognized HIV-1 Gag peptides based on South African Du422 and Chinese subtype C peptide reagents (Figure 3.6).

There was a wide range of HIV-specific T cell responses among different individuals. The magnitude of HIV-specific T cell responses ranged from 0-19843 net SFU/10^6 PBMC. There were 15 individuals who had cumulative responses >5 000 SFU/10^6 PBMC, 11 individuals had 1 000-5 000 SFU/10^6 PBMC and 12 had <1 000 SFU/10^6 PBMC to $C_{S.A}$ Gag peptide pools (Figure 3.6). A similar trend was observed for the C_{CH} peptide pools, with 15 individuals had cumulative responses > 5 000 SFU/10^6 PBMC, 14 individuals having 1000-5 000 SFU/10^6 PBMC and nine having <1 000 SFU/10^6 PBMC to C_{CH} Gag peptide pools. For the remaining subtypes tested, there were fewer high magnitudes of responses. Subtype D peptide pools had the highest number of individuals with low magnitudes of T cell responses (16 individuals with <1000 SFU/10^6 PBMC).

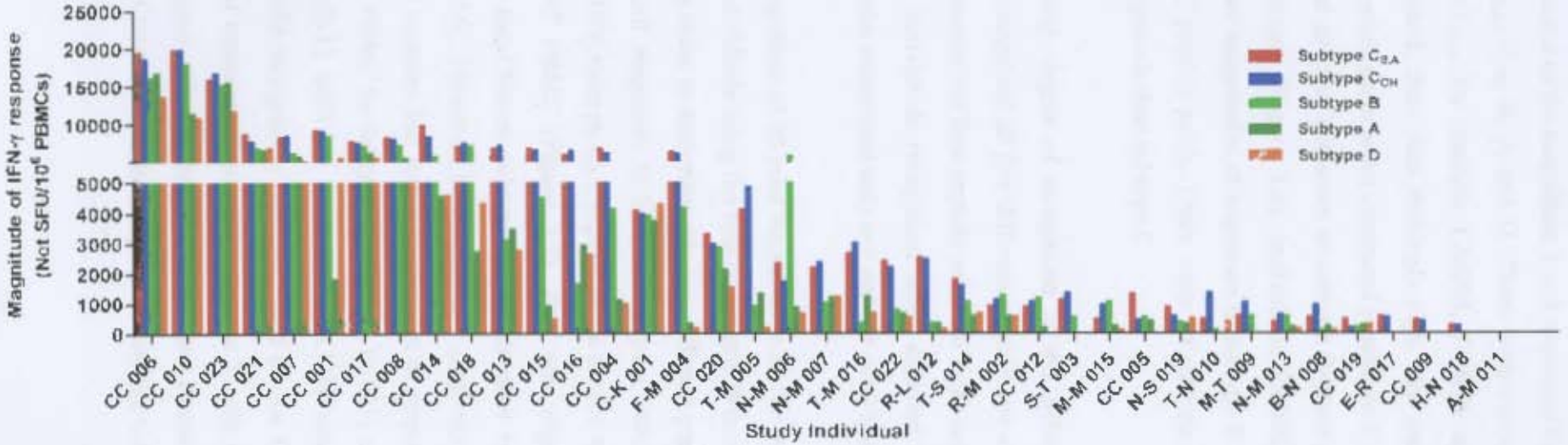


Figure 3.5 The total magnitude of Gag response to subtypes A, B, $C_{S.A}$, C_{CH} and D. The responses shown are the total magnitudes of IFN-γ producing HIV-specific T cells as detected by the ELISpot-assay among 39 participants, expressed as the number of net SFU/10^6 PBMC for the different subtype peptide reagents, for each study individual (see colour key).

The general trend of the magnitude T cell responses for the different subtypes was in the order $C_{S.A}$, C_{CH}, B, A and D. There were cases when C_{CH} were higher than responses to $C_{S.A}$, for example T-M005, T-M016 and T-N010 (Figure 3.6). In a single individual, there was strikingly high total magnitude of responses to non-subtype C peptide pools when compared to subtype C peptide pools. This individual, N-M006, had stronger responses to subtype B-based peptide pools when compared to other subtypes (Figure 3.6). Individuals R-M002, CC012 and M-M015 had slightly higher magnitudes of responses to subtype B peptide pools when compared to subtype C peptide pools. There were no subtype A and D responses that were higher in magnitude than subtype C.

There was high degree of recognition of the different subtypes. Thirty (78.9%) individuals recognized all five different peptide sets used (Figure 3.6). Three (7.9%) individuals recognized four peptide sets, and same applied to two peptide sets. Only two (5.3%) individuals recognized three different peptide sets. There were no individuals who recognized only one peptide set (Figure 3.6).

The total magnitude of all pool responses to all the five peptide sets were compared for all the individuals using the non-parametric Wilcoxón signed rank test. This was performed in order to determine whether different peptide sets can elicit equal HIV-specific T cell responses in HIV-1 subtype C-infected individuals. The median response to HIV subtype $C_{S.A}$ peptide pools for the study subjects was 2 530 (0-19 817) SFU/10^6 PBMC (Figure 3.7). This was slightly higher than the median response to the Chinese subtype C peptide pool reagents, at 2 470 (0-19 843) SFU/10^6 PBMC. However, HIV-specific T cells recognizing subtypes B, A and D peptide pool reagents had lower magnitudes of responses, namely 1 175 (0-17872) net SFU/10^6 PBMC for subtype B, 868.3 (0-16 723) net SFU/10^6 PBMC for subtype A and 560 (0-13 605) net SFU/10^6 PBMC for subtype D. In most cases, HIV-specific T cells recognizing peptides based on the $C_{S.A}$ and C_{CH} gave the highest magnitude of responses. Thus, HIV-specific T cells recognizing peptides based on the heterelogous subtype, that is subtypes not responsible for infection, had lower magnitude of responses when compared to subtype C-based peptide reagents.

There was no statistically significant difference in the magnitude of HIV-specific T cells against subtype $C_{S.A}$ and subtype C_{CH} peptide reagents ($p = 0.8363$, Figure 3.7). Subtype $C_{S.A}$ and subtype B peptide reagents' responses differed significantly ($p < 0.0001$). Statistically significant differences were also obtained between the magnitude of HIV-specific T cells recognizing subtype $C_{S.A}$ peptide reagents and subtypes A and D ($p < 0.0001$). In addition, the magnitude of responses against subtype B peptide reagents was significantly higher than that of both subtype A ($p = 0.0020$) and subtype D ($p < 0.0001$).

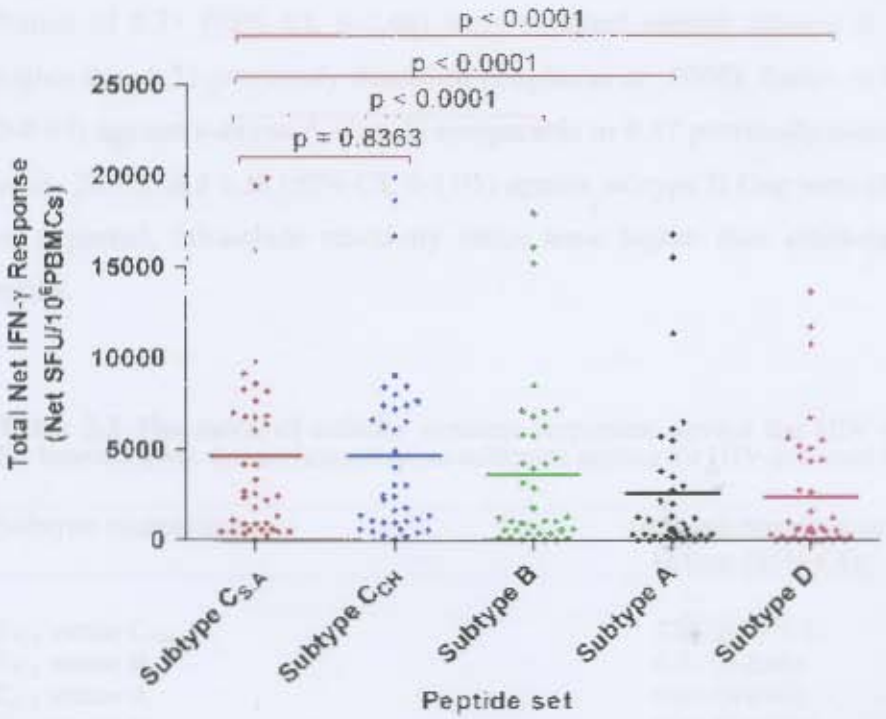


Figure 3.6 Recognition of the subtypes A, B C_{Du422}, $C_{Chinese}$ and D Gag sequences in HIV-1 subtype C-infected individuals. Each dot represents the total magnitude of Gag-specific T cell responses to each subtype in each individual. P values indicate significance of differences between groups

3.3.3.2 Cross-reactivity of T cell responses to the major HIV-1 subtypes

A quantitative assessment of the ratios of ELISpot responses to the four HIV-1 subtypes tested in the study is presented in Table 3.2. The subtype from South Africa ($C_{S.A}$), was used as the denominator of the ratio and each of the other three subtypes and the other subtype C was used separately in the numerator. The ratios

obtained are referred to as cross-clade reactivity ratios. Therefore, the ratio of subtype $C_{S.A}$ versus subtype $C_{S.A}$ is one, showing equal recognition of Gag peptides based on these HIV-1 variants by HIV-specific T cells.

The mean ratio of HIV-specific T cell responses for all the 39 study subjects was 1.08 (95% confidence interval [CI], 0.34-2.8) against subtype $C_{S.A}$ Gag versus subtype C_{CH} Gag, showing that C_{CH} had a slightly higher total magnitude of responses than $C_{S.A}$. Ratios to other HIV-1 subtypes were lower than ratios within subtype Cs, showing that subtype C-infected individuals recognize peptides based on other subtypes with a lower magnitude than they do to clade-matched peptides. Ratios of 0.71 (95% CI, 0-2.48) were obtained against subtype B Gag, slightly higher than 0.51 previously described (Coplan *et al.*, 2005). Ratios of 0.41 (95% CI, 0-0.97) against subtype A Gag, is comparable to 0.47 previously described (Coplan *et al.*, 2005), and 0.38 (95% CI, 0-1.05) against subtype D Gag were obtained. Thus, as expected, intra-clade reactivity ratios were higher than cross-clade reactivity ratios.

Table 3.2 The ratios of cellular immune responses against the HIV-1 Gag protein for heterelogous versus homologous subtypes among 39 HIV-infected individuals

Subtype comparison	Immune response ratio (Mean (95% CI))
$C_{S.A}$ versus C_{CH}	1.08 (0.34-2.8)
$C_{S.A}$ versus B	0.71 (0-2.48)
$C_{S.A}$ versus A	0.41 (0-0.97)
$C_{S.A}$ versus D	0.38 (0-1.05)
Previously reported for South Africa*	
A versus C	0.47 (0.38-0.57)
B versus C	0.51 (0.40-0.66)

*Coplan *et al.*, 2005

The cross-reactivity of HIV-specific T cells to the Gag protein was investigated in a more graphic way. Figure 3.8 shows Log_{10}-transformed ELISpot HIV-specific T cell responses against the Gag protein. The slope of the line of best fit is approximately 1, making an angle that is approximately 45^0 to the x-axis. This line represents cross-clade reactivity ratio of 1

Generally, there was a strong correlation of Log_{10}-transformed ELISpot responses between the subtype $C_{S.A}$ and the four other HIV-1 subtypes used (Figure 3.8). Most data points cluster around this line, illustrating this high degree of intra- and cross-clade recognition. The highest correlation was obtained for responses to the same subtype, between subtype $C_{S.A}$ and subtype C_{CH} ($r = 0.9728$, $p < 0.0001$), where most responses clustered closely around the 45^0 line (Figure 3.8). Subtype $C_{S.A}$ and D had the lowest cross reactivity correlation ($r = 0.8595$, $p < 0.0001$), where it is clearly seen that data point are more dispersed and further away from the 45^0 line.

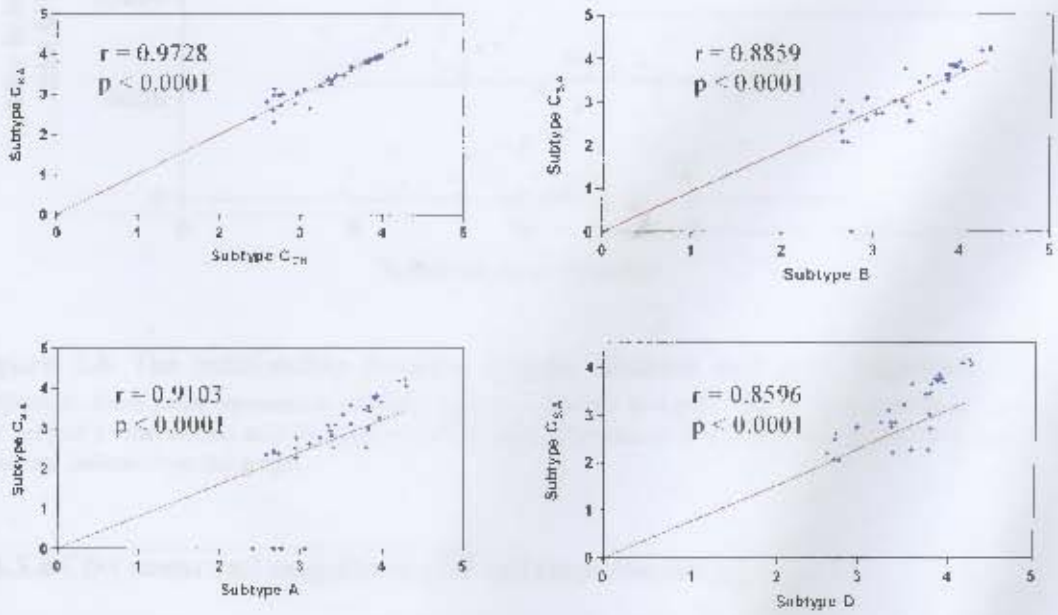


Figure 3.7 Cross-reactivity of T cell immune responses against HIV-1 Gag. Values on the axes represent the net number of IFN-γ-expressing T cells/10^6 PBMC. Responses with zero net SFU/10^6 PBMCs were assigned a value of 1. The r value is the non-parametric Spearman correlation and the p value given 95% significance level.

3.3.3.3 Genetic distance and HIV-specific T cell responses

The amino acid distance between an individual's infecting viral Gag protein sequence and the peptide sequence were determined. This was plotted against the total magnitude of IFN-γ response to the particular peptide variant set in order to determine the impact genetic distance has on the recognition of Gag peptides by HIV-specific T cells. There was no significant correlation between the magnitude of response and genetic distance between the infecting viral sequence and the ELISpot peptide reagent sequence ($r = -0.0042$, $p = 0.9670$) (Figure 3.9).

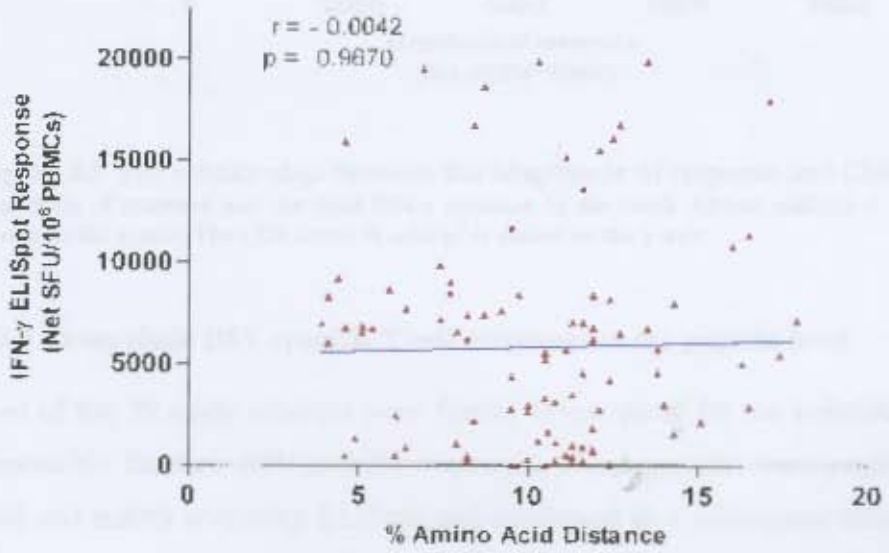


Figure 3.8 The relationship between genetic distance and total magnitude of response. Each point represent a subject's total net response to a particular subtype peptide set and that subject's virus amino acid distance from that peptide sequence. The Spearman correlation r and p value are indicated on the graph.

3.3.3.4 CD4 count and magnitude of T cell response to Gag

The relationship between the total magnitude the IFN-γ T cell responses to Gag peptide pools based on the South African subtype C protein sequence and $CD4^+$ T cell count was determined for all study participants using Spearman correlation analyses, to determine whether the magnitude of IFN-γ responses is a correlate of

disease progression. There was a negative trend obtained, although this was not statistically significant ($r = -0.074$, $p = 0.714$) (Figure 3.10).

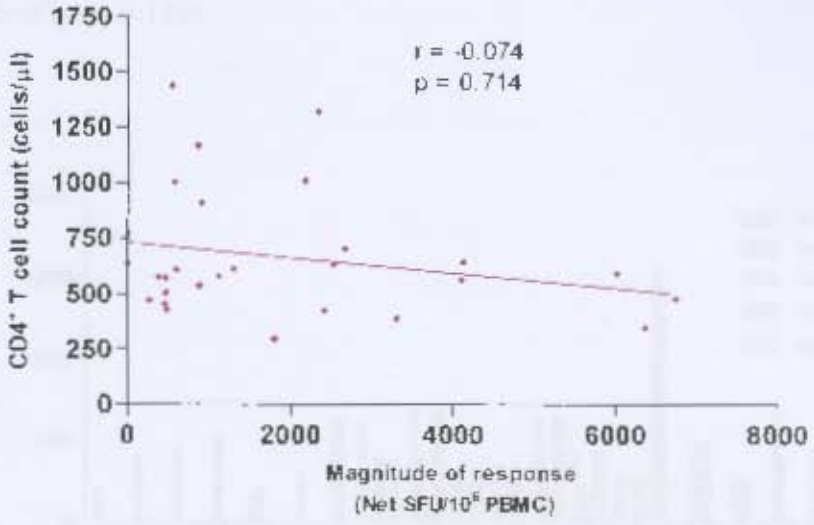


Figure 3.9 The relationship between the Magnitude of response and CD4 count. The magnitude of response was the total IFN-γ response to the South African subtype C peptide pools shown on the x-axis. The CD4 count in cells/μl is shown on the y-axis.

3.3.4 Cross clade HIV-specific T cell responses at the peptide level

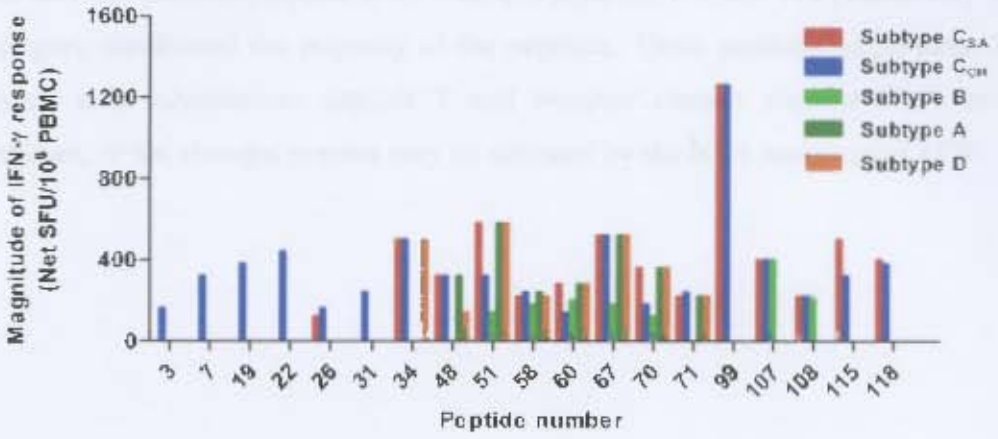
Two of the 39 study subjects were further investigated for the individual peptides responsible for their HIV-specific responses. These peptides were predicted by the pool and matrix screening ELISpot and confirmed in a subsequent assay. The two individuals CC22 and CC07 were chosen because of their broad predicted reactivity. Due to time constraints, only these individuals' single peptide responses were performed.

3.3.4.1 Evaluation of cross-clade T cell responses to HIV-1 Gag

The magnitude of responses contributed by each reactive peptide from each of the five peptide sets for study individual CC22 is shown in Figure 3.11a. When there were adjacent reactive peptides, the peptide with the highest response was considered the reactive peptide. In the case of three consecutive peptides being reactive, two peptides were considered positive. Individual CC22 recognized a total of 19 peptides. Peptide 99 had the highest magnitude of response, and was

recognized in two peptide sequence variants, C_{SA} and C_{CH}. This peptide is highly conserved among the subtype C peptide sequence variants tested (Table 3.3). C_{CH} peptides had the highest percentage recognition (34%) compared to other peptide variants (Figure 3.11b).

(a)



(b)

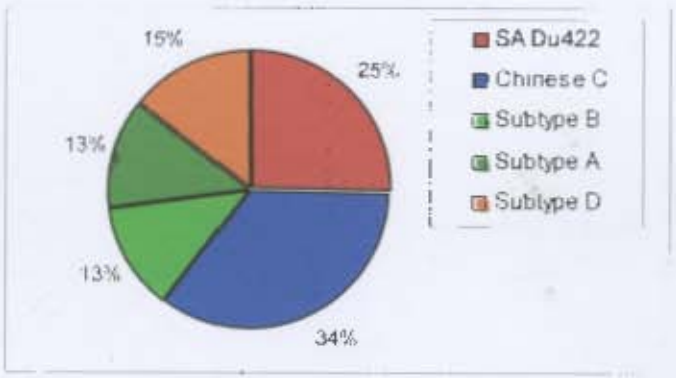


Figure 3.10 Assessment of HIV-1-specific T cell responses cross-reactive with the clades A, B, C (South African C and Chinese C) and D sequences at the single peptide level.**(a)** The recognition of peptides from subtypes A, B, C (South African C and Chinese C) and D in study individual CC22. **(b)** The percentage of HIV-specific T cells recognizing each HIV-1 subtype in individual CC22. The magnitude of response is expressed as the numbers of IFN-γ-producing cells/10^6 PBMC. Peptide numbers are according to the South African Du422 (C_{SA}) peptide reagent numbers. S.A Du422 is the C_{SA} and Chinese C is the C_{CH}.

Peptide sequences were compared to the infecting virus sequence (Table 3.3). The reactive peptides in individual CC22 could be divided into different categories.

There were some reactive peptides which had the same sequence as the infecting viral sequence, for example peptides 19, 48, 67 and 71. Peptides 3, 7 and 19 show the position of either HLA anchor residues or T cell receptor contact sites. These peptides had peptide variants that had single amino acid changes that lead to loss of recognition by HIV-specific T cells. Another category of peptides was those peptides which had amino acid sequences different from the infecting viral sequence but still had positive responses, for example peptides 115 and 118 (Table 3.3). This category constituted the majority of the peptides. These peptides might have their amino acid substitutions outside T cell receptor contact sites or HLA anchor residues, or the changes present may be tolerated by the HLA molecule of TCR.

Table 3.3 The recognition of the subtypes A, B, C and D peptide sequences in individual CC22

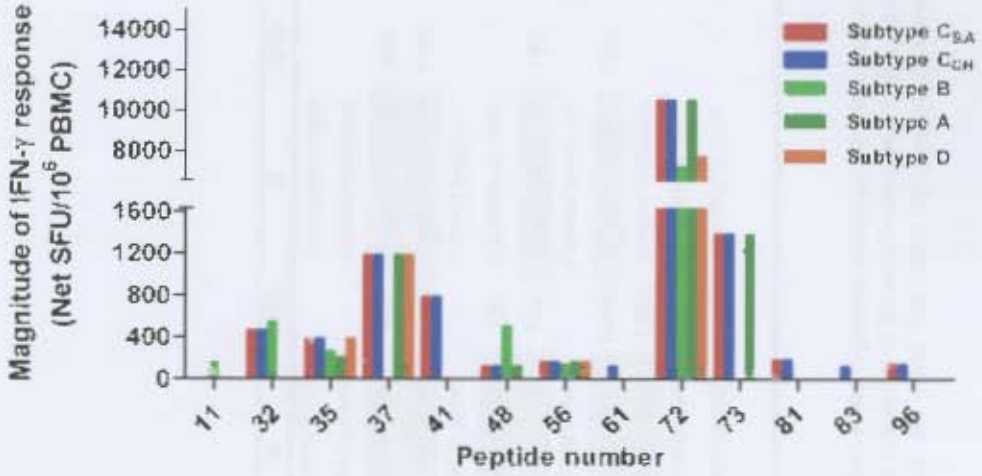
Peptide no	Location*	$C_{S.A}$	SFU	C_{CH}	SFU	B	SFU	A	SFU	D	SFU	Infecting seq
C1-3/C2-3/B-3/A-6*/D-6	12-24 (p17)	GEKLDKWEKIRLRPG		GGKLDKWEKIRLRPG	160	SGGELDRWEKIRLRP		GGKLDAWEKIRLRPG		GGKLDAWEKIRLRPG		GEK_DKWEKIKLRPG
C1-7/C2-7/B8/A65/D65	21-42 (p17)	KKHYMLKHIVWASRE		KKHYMLKHLVWASRE	320	IKKKYKLKHVVWASR		KKKYHLKHLVWASRL		KKKYRLKILVWASRL		KKPYMHKHLVWASRL
C1-15/C2-19/B-15/A-77/D-77	74-88 (p17)	ELKSLYNTVATLYCV		ELRSLRNTVATLYCV	380	LLLRSLYNTVATLYC		LKSLYNIVATLYCVH		LLRSLYNIVAILYCV		ELPSLRNIVAILYCV
C1-22/C2-22/22/A-80/D-80	86-101 (p17)	YCVHEKIEVRDTKEA		YCVHEQIEIRDTKEA	440	LYCVHQRIEVKDIKL		CVHQRDVKDIKLA_		YCVIIEHEVKDIKLA		YCVHAKIDVRDIKLA
C1-26/C2-26/B-26/A-84/D-84	103-121 (p17)	DKIEELQNKSQQKTQ	120	DKILEEQNKGQGKIQ	160	LEKIFFFQNKSKKKA		KIEFQNKSKQKTQQ		KKMFEFQNKSKKKAQ		DKIFFFQNKSQQKTQ
C1-31/C2-31/B-31/A89/D-89	127-153 (p17/p24	DGKVSQNYPIVQNLQ		DLKVSQNYPIVQNLQ	240	DTQNSSQVSQNYPIV		TQSSKVSQNYAIKHP		TQNSSQVSQNYPIVQ		KGKVSQNYPVQNSQ
C1-34/C2-34/B-35/A-2/D-2	152-167 (p24)	LQGQMVHQAISPRIL	500	LQGQMVHQAISPRIL	500	NLQGQMVHQAISPRT		AQGQMHQAISPRT_		LQGQMVHQAISPRT_	500	SQGQMVHQAISPRT_
C1-48/C2-48/B-48/A-16/D-16	158-177 (p24)	IIQAAMQMLKDTINE	320	HQAAMQMLKDTINEE	320	GIIQAAMQMLKFTNL		HQAAMQMLKDTINFF	320	IIQAAMQMLKFTNLL	140	HQAAMQMLKDTINEE
C1-51/C2-51/B-52/A-19/D-19	221-236 (p24)	INEEAAEWDRLHPVHA	580	INFEAAFWDRVHPVHA	320	INEEAASWDRLHPVH	140	INEEAAEWDRLHPVHA	580	INEEAAEWDRLHPVH-A	580	INEEAASWDR_HPVQA
C1-58/C2-58/B-59/A-26/D-26	250-264 (p24)	SDIAGTTSTLQEQIA	220	SDIAGTTSTLQTQIA	240	GSDIAGTTSTLQEQ	180	SDIAGTTSTLQEQIG	240	SDIAGTTSTLQEQIA	220	SDIAGTTSTLQEQIV
C1-60/C2-60/B-61/A-28/D-28	258-272)p24)	TLQFQIAWMTSNPP	280	TLQCQIAWMTSNPPV	140	RTLQLQIGWMTNNPP	200	ILQLQIGWMISNPPI	280	ILQLQIAWMISNPPI	280	ILQLQIVWVISNPPI
C1-67/C2-67/B-68/A-35/D-35	287-301 (p24)	LNKIVRMYSPVSILD	520	LNKIVRMYSPVSILD	520	GLVKIVRMYSPISIL	180	LNKIVHMYSPVSILD	520	LNKIVRMYSPVSILD	520	LNKIVRMYSPVSILD
C1-70/C2-70/B-71/A-38/D-38	299-313 (p24)	ILDIRQGPKEPFRDY	360	ILDIKQGPKEPFRDY	180	NLDIRQGPKEPFRD	120	ILDIRQGPKEPFRDY	360	ILDIRQGPKEPFRDY	360	ILDIKQGPKEPFRDY
C1-71/C2-71/B-72/A-39/D-39	303-317 (p24)	RQGPKEPFRDYVDRF	220	KQGPKLPFRDYVDRF	240	IRQGPKEPFRDYVDR		RQGPKEPFRDYVDRF	220	RQGPKEPFRDYVDRF	220	KQGPKLPFRDYVDRF
C1-99/C2-99/B-100	428-442 (p7)	HIARNCRAPRKKGCW	1260	HIARNCRAPRKKGCW	1260	KPCHIAKNCRAPRKK						IILAHNCRAPHKKGCW
C1-107/C2-107/B-109	452-467 (p1)	FLGKIWPSHKGRPGN	400	FLGKIWPSHKGRPGN	400	FLGKIWPSHKGRPGN	400					FLGKIWPSYKGHPGN
C1-108/C2-108/B-110	456-471 (p1/p6)	IWPSHKGRFGNFLQS	220	IWPSHKGRFGNFLQS	220	IWPSHKGHPGNFLQS	220					IWPSYKGRPGNFLQS
C1-115/C2-115/B-117	505-520 (p6)	FEETTPAPKQEPIER	500	FLLTIPAPKQEPKDR	320	FGLLTIIPSQKQLPI						FEETTPAPKQELKDR
C1-118/C2-118/B-120	518-533(p6)	IEREPLTSLKSLFGS	400	KDREPLTSLKSLFGS	380	KELYPLASLRSLFGN						KDRPPLTSLKSLFGS

*The underlined sequences represent the overlapping part for those peptides that had an overlapping reactive peptide. The letters in red represent amino acid differences when compared to the subtype $C_{S.A}$ sequence. The blue letters in the infecting viral sequence represent amino acid differences when compared to the peptide sequence that gave the highest responses when compared to other corresponding peptides in the five peptide sets. Peptides highlighted in grey are those that came up positive (reactive).

For study subject CC07, a total of 15 peptides were recognized (Figure 3.12a). Peptide 72 was the most immunodominant and cross-reactive peptide for individual CC07, and together with other cross-reactive peptides, 37, 56 and 73, had high degrees of sequence similarity among their corresponding variants as well as to the infecting viral sequence (Figure 3.12a and Table 3.4). Subtype C peptides had the highest percentage recognition compared to heterelogous peptides (Figure 3.12b), with C_{CH} again having higher recognition than $C_{S.A}$.

Peptide variants with similar sequence to the infecting viral sequence can be identified from Table 3.4. These are peptides 41, 56, 72, 73, 81 and 96. An interesting observation was the recognition of a peptide variant with a sequence different from the infecting viral sequence, while the corresponding peptide sequence with the same sequence as the infecting viral sequence was not recognized, as is the case for peptides 11 and 83. The subtype B variant of peptide 11 had positive response but its sequence differs from the infecting viral sequence while the two subtype C peptides had the same sequence as the infecting viral sequence, but were not recognized. For peptide 83, the $C_{S.A}$ had the same sequence as the infecting viral sequence but was not recognized, while the C_{CH} peptide was reactive, but had a single amino acid change from the infecting viral sequence. This suggests escape from T cell immune responses. It is possible that the reactive peptide sequence is similar to the sequence *which HIV-specific T cells initially encountered, which mutated to the current* infecting viral sequence due to immune pressure. No HIV-specific T cells are present to the current epitope as it may no longer be able to bind the MHC molecule or TCR contact sites, however, a weak memory T cell responses still exists specific for the previous epitope sequence.

(a)



(b)

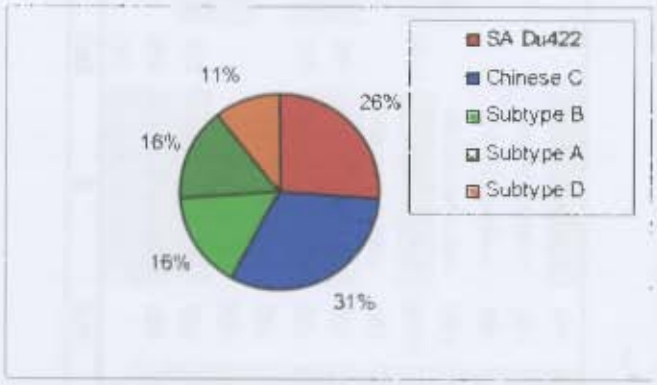


Figure 3.11 Assessment of HIV-specific T cell responses cross-reactive with the subtypes A, B, C (South African C and Chinese C) and D sequences at the single peptide level.(a) The recognition of peptides from clades A, B, C (C_{SA} and C_{CH}) and D in study individual CC07. (b) The percentage of HIV-specific T cells recognizing each HIV-1 subtype in individual CC 07. The magnitude of response is expressed as the numbers of INF-γ-producing cells/10^6 PBMC. Peptide numbers are according to the C_{SA} peptide reagent numbers. S.A Du422 is the C_{SA} and Chinese C is the C_{CH}.

Table 3.4 The recognition of the subtypes A, B, C and D peptide sequences in individual CC07

Peptide no	Location*	C_{SA}	SFU	C_{CH}	SFU	B	SFU	A	SFU	D	SFU	Infecting seq
C1-1/C2-1/B1/A53/D53	47-56 (p17)	ERFALNPGLLETSEG		ERFALNPGLLETSEG		LEHFA NPGLLETSE	160	ERFALNPGLLETAEG		ERFALNPGLLETSEG		LEKFALNPGLLETSG
C1-32/C2-32/B32/A90/D90	138-153 (p24)	SQNYPVVQNLQGQMV	460	SQNYPIVQNLQGQMV	460	SSQMSQNYPIVQNLQ	540	QNSQNYALKHHAYSL		SQVSQNYPIVQNLQG		SQNYPIQNLQGQMV
C1-35/C2-35/B-36/A-3/D-3	156-171 (p24)	MVHQAISPRTLNAWV	380	MVHQAISPRTLNAWV	380	QMVHQAISPRTLNAW	260	QQMHQALSPRTLNAWV	200	MVHQAISPRTLNAWV	380	MVHQPISPRTLNAVV
C1-37/C2-37/B-38/A5/D5	164-180 (p24)	RTLNAWVKVIEEKAF	1180	RTLNAWVKVIEEKAF	1180	HRTLNAWVKVVEEKA		RTLNAWVKVIEEKAF	1180	RTLNAWVKVIEEKAF	1180	RTLNAWVKVIEEKAF
C1-41/C2-41/B-42/A9/D9	182-191 (p24)	PEVIPMFTALSEGAT	780	PEVIPMFTALSEGAT	780	QPEVIPMFTALSEGA		PEVIPMFTALSEGAT		PEVIPMFTALSEGAT		PEVIPMFTALSEGAT
C1-48/C2-48/B-49/A-18/D-1	158-177 (p24)	HQAAMQMLKDTINEE	120	HQAAMQMLKDTINEE	120	GHQAAMQMLKDTINE	500	LHQAAMQMLKDTINEE	120	HQAAMQMLKDTINEE		GHQAAMQMLKDTINE
C1-56/C2-56/B57A74D24	247-256 (p24)	GQMREPRGSDIAGTT	160	GQMREPRGSDIAGTT	160	PGQMREPRGSDIAGT	140	QQMHEPNGSDIAGTT	160	GQMREPRGSDIAGTT	160	GQMREPRGSDIAGTT
C1-61/C2-61/B62/A23/D23	261-271(p24)	EQIAWMTSNPPIPVGD		GQIAWMTSNPPIPVGE	120	EQIAWMTSNPPIPVG		EQIAWMTSNPPIPVGD		EQIAWMTSNPPIPVGD		EQIAWMTSNPPIPVGD
C1-72/C2-72/B-73/A-40/D-4	317-321 (p24)	KEPFRDYVDRFFKTL	10480	KEPFRDYVDRFFKTL	10480	FKEPFHDYVDRFYKT	7160	KEPFRDYVDRFFKTL	10480	KEPFHDYVDRFYKTL	7670	KEPFRDYVDRFHKTL
C1-73/C2-73/B-74/A41/D41	311-324 (p24)	RDYVDRFFKTLRAEQ	1380	RDYVDRFFKTLRAEQ	1380	FRDYVDRFYKTLRAE		RDYVDRFFKTLRAEQ	1380	RDYVDRFYKTLRAEQ		RDYVDRFHKTLRAEQ
C1-81/C2-81/B-82/A49/D49	344-358 (p24)	ANDPDKTILKALGPG	180	ANDPDKTILKALGPG	180	NANDPDKTILKALGP		ANDPDKTILRALGPG		ANDPDKTILRALGPG		ANDPDKTILRALGPG
C1-83/C2-83/B-84/A51/D51	352-361 (24)	LRALGPGATLEEMMT		LRALGPGATLEEMMT	120	LKALGPAATLEEMM		LRALGPGATLEEMMT		LKALGPGATLEEMMT		LRALGPGATLEEMMT
C1-86/C2-86/B-87	411-420 (?)	RIVKCFNCGKEGHIA	140	RIVKCFNCGKEGHIA	140	NPPIPVKQFNCGKEG						RIVKCFNCGKEGIIA

*The underlined sequences represent the overlapping part for those peptides that had an overlapping reactive peptide. The letters in red represent amino acid differences when compared to the subtype C_{SA} sequence. The blue letters in the infecting viral sequence represent amino acid differences when compared to the peptide sequence that gave the highest responses when compared to other corresponding peptides in the five peptide sets. Peptides highlighted in grey are those that came up positive (reactive).

3.3.4.2 Breadth of HIV-specific T cell responses

The use of different peptides sets to test for T cell immune responses was investigated to determine whether extra responses could be detected when additional peptide variant sets are used in an ELISpot assay. The use of a number of peptide sets increased the number of responses detected, as shown in Table 3.5 for two individuals. The addition of a variant subtype C peptide set is mainly responsible for this effect. No additional T cell responses were detected in these two study individuals by the addition of subtype D Gag peptides.

Table 3.5 Peptide sets and number of responses detected

Study Individual	$C_{S.A}$	$C_{S.A}$+C_{CH}	$C_{S.A}$+C_{CH}+B	$C_{S.A}$+C_{CH}+B+A	$C_{S.A}$+C_{CH}+B+A+D
CC22	14	19	19	20	19
CC07	10	12	13	13	13

3.3.4.3 Distribution of T cell responses among HIV-1 Gag regions

For each peptide set, a peptide with a different sequence from others in the same set was considered a different peptide. After consideration of common peptides among the five peptide variants, there were 30 reactive peptides recognized in the two individuals. The distribution of the responses among the different Gag regions is shown in Table 3.6. Twenty percent (6/30) of the peptides recognized were from the p17 region. The more conserved region of Gag, p24 contributed the majority of the peptides recognized (60%, 18/30 peptides). The last part of Gag protein (p15), which is the most variable, contributed the same number of peptides as p17 in these two individuals (20%, 6/30 peptides).

Table 3.6 T cell recognition of different HIV-1 Gag protein regions

Protein	No of peptides	Overall % recognition
p17	6	20
p24	18	60
p15	6	20

Some peptides had high magnitude of responses and a peptide which had >1 000 SFU/10^6 was empirically classified as an immunodominant peptide. Four immunodominant

peptides were identified in the two study subjects, and the distribution of these within the Gag protein is shown in Figure 2.12. Two of the four peptides were from the p24 region.

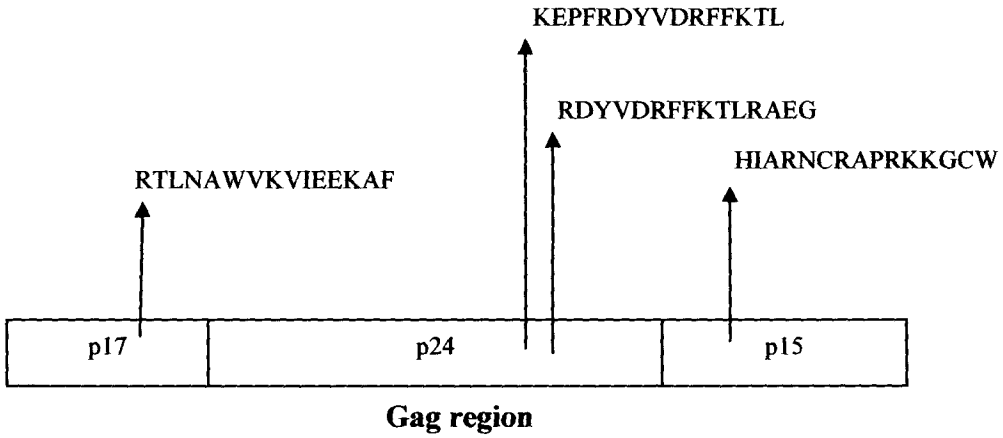


Figure 3.12 The distribution of immunodominant epitopes over the HIV-1 Gag protein. Confirmatory ELISpot analysis was performed on two subjects to determine the reactive individual peptides. Shown is the distribution of immunodominant peptides found in two individuals as well as their sequences. Immunodominant peptides were defined as those peptides giving a net IFN-γ ELISpot response of > 1000 SFU/10^6 PBMC. The heights of the arrows are proportional to the magnitude of net IFN-γ response to the peptide.

The peptides which were classified as the immunodominant peptides in the two individuals were further analyzed for previously defined epitopes using the Epitope Location Finder tool of the Los Alamos Immunology Database (http://www.hiv.lanl.gov/content/hiv-db/ELF/epitope_analyzer.html). These peptides had high epitope density and most of the epitopes were defined in individuals with known HLAs (Table 3.7). Peptides 72 and 73 had the highest number of epitopes previously defined. The epitopic sequences common to peptides 72 and 73 were listed under peptide 72 and left out under peptide 73. Most of the epitopes were restricted by HLA-B alleles.

Table 3.7 $CD8^+$ T cell epitopes within immunodominant peptides

Peptide No HLA	peptide sequence (epitope sequence)	SFU/10^6 PBMCs	References
C 37	RTLNAWVKVIEEKAF	1180	
A2	RTLNAWVKV------		Cobert et al., 2003
A*0201	-TLNAWVKW------		Lorin et al., 2005a
A*02	-TLNAWVKVV-----		Rinaldo et al., 2000
A2	-TLNAWVKVV-----		Missale et al., 2004
A2, A*0202	-TLNAWVKVV-----		Rowland-Jones et al., 1998
A2	-TLNAWVEV1-----		Boleste et al., 2005a
	---NAWVKIEEK--		Jones et al., 2004
	---NAWVKVVEEKA-		Amara et al., 2005a
B*1503	------VKVIEEKAF		Kiepiela et al., 2007
C 72	KEFERDYVDRFFKTL	10480	
A*0201	-EFERD?VDR?----		Thakar et al., 2005
	--FRDYVDRFF---		Masemola et al., 2004a
Cw*1801	---FRDYVDRFF---		Kiepiela et al., 2007
Cw*18	---FRDYVDRFF---		Frahm et al., 2007
B*1801	---FRDYVDR?YK--		Liu et al., 2006
	---FRDYVDRFYK--		Kaul et al., 2001
B18	---FKDYVDRFYK--		Wee et al., 2002
'B44, A26, B70	----RDYVDRFYKTL		Ogg et al., 1998b
'B*4402	----RDYVDRFYKTL		Frahm et al., 2007
'A*2402	----RDYVDRFYKTL		Dorrell et al., 1999
'B44	----RDYVDRFYKTL		Draenert et al., 2004b
'A24	----RDYVDRFYKTL		Kaul et al., 2001a
'A24_5-14	-----DYVDRFYKTL		Jones et al., 2004
'A*2402	-----DYVDRFYKT-		Ferrari et al., 2000
'A26, B70	-----YVDRFFKTL		Dorrell et al., 2001
'B*1510, B70	------YVDRFFKRL		Novitsky et al., 2001
'B70	------YVDRFYKTL		Ferrari et al., 2000
'A*0207	------YVDRFYKTL		Currier et al., 2002b
'Cw*0303	------YVDRFFKTL		Frahm et al., 2007
'Cw*0304	------YVDRFFKTL		Kiepiela et al., 2007
'B*1503	------YVDRFFKTL		Masemola et al., 2004a
C 73	RDYVDRFYKTLRAEQ	1380	
B14	DRFYKTLRA--		Cao et al., 1997
B14	----DRFWKTLRA--		Kaul et al., 2002
B*1402	----DRFYKTLRA--		Frahm et al., 2007
B14, B*1402	----DRFYKTLRA--		Rowland-Jones et al., 1998
B14	----DRFYKTLRA--		Rowland-Jones et al., 1999
B14	----DRFFKTLRA--		Kiepiela et al., 2004
B*1403	----DRFFKTLRA--		Masemola et al., 2004a
B*1401	----DRFYKTLRA--		Kiepiela et al., 2007
B*1402	----DRFFKTLRA--		Boutwell et al., 2007
B14	----DRFYKTLRAE-		Wagner et al., 1999
B14	----DRFYKTLRAE-		Musey et al., 2003
B*1402	----DRFYKTLRAEQ		Jones et al., 2004
C 99	KIAMYCRAFRKKTCW	1260	
A3	-LAMYCRAFRK-----		DeGroot et al., 2003
B14	CRAFRKKTC-		Frahm et al., 2007

Bold amino acids represent the difference between peptide sequence used in ELISpot assay and previously defined epitope

3.3.4.4 Variability of cross-reactive HIV-1 Gag peptides

To more closely characterize the viral regions that were preferentially cross-recognized by HIV-1-specific T cells, we classified all the 32 overlapping peptide sets from the two confirmed subjects according to recognition of one, two, three, four or five peptide variants. The median Shannon entropy scores of the peptides in these two different groups were determined (Figure 3.14 a). Entropy values ranged from 0-1.514 for all the peptide sets. Peptides recognized in one sequence variant (8 peptides) were more than those recognized in each of the 2, 3, 4 and 5 sequence variants (7, 6. 4 and 7, respectively).

The median Shannon entropy score of peptides recognized in one and two peptide variants was 0.94 (range 0.09-1.26) and 0.44 (range 0.08-1.51), respectively. As shown in Figure 3.14a, these peptide variants had significantly higher entropy scores when compared to peptide variants recognized in three, four and five sequence variants, whose median Shannon entropy scores were 0.19 (range 0-0.45), 0.08 (range 0.08-0.11) and 0.06 (range 0.05-0.09), respectively ($p < 0.05$, Mann-Whitney test). There was no significant difference between the entropy score of peptides recognized in one sequence variant and those recognized in two sequence variants ($p = 0.3969$). The same was true for peptides recognized in four sequence variants and five sequence variants ($p = 0.6551$).

The percentage homology for all the five peptide variants ranged from 58.6% to 100%. The degree of inter-clade homology, defined as the degree of sequence similarity between the different clades A, B, $C_{S\,A}$, C_{CH} and D sequence variants, was highest in the subset of peptides recognized in all five sequence variants (Figure 3.14 b). Peptides recognized in three, four and five sequence variants had significantly higher percentage homology when compared to peptides recognized in one and two sequence variants ($p<0.05$). There was no significant difference between percentage homology of peptides recognized in one and two sequence variants ($p = 0.7263$) (Figure 3.15b).

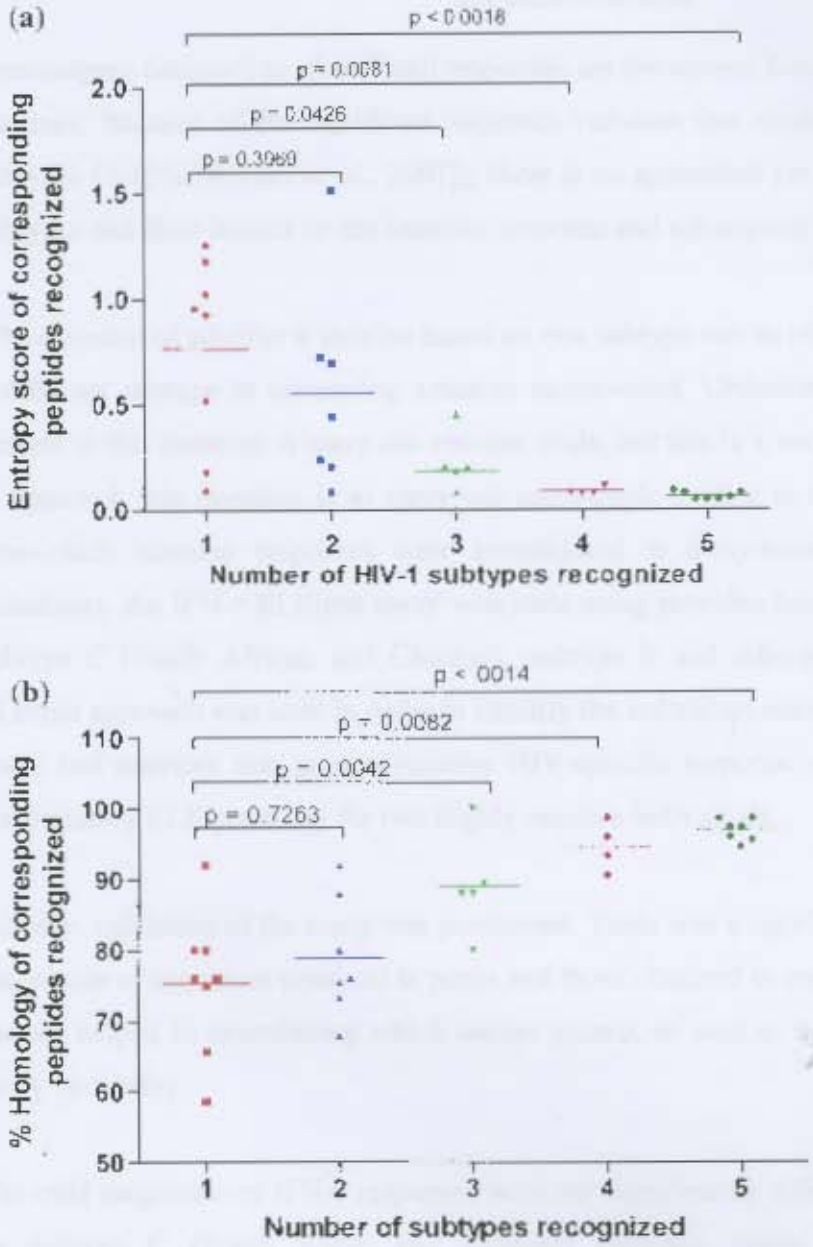


Figure 3.13 Characterization of viral peptides with intra- and inter-clade cross recognition by HIV-specific T cells. (a) Significantly lower entropy of peptides recognized in at least three subtype sequences. (b) Significantly higher inter-clade homology of peptides recognized in more than two clade-specific sequences. Average Shannon entropy scores for peptides of the five different HIV peptide sets used in the ELISpot assay are shown. Peptides were categorized according to the number of HIV-1 subtypes in which each of the peptide was recognized. Different categories were compared using the non-parametric Mann-Whitney test.

3.4 DISCUSSION

Immunogens designed to elicit T cell responses are the current focus in the development of HIV vaccines. Because of the significant sequence variation that exists between and within HIV-1 subtypes (7-15% [Korber *et al.*, 2001]), there is no agreement yet as to the importance of HIV subtypes and their impact on the immune response and subsequent efficacy of vaccines.

The question of whether a vaccine based on one subtype can be effective in a population where a different subtype is circulating remains unanswered. Ultimately, the best way to have an answer to this question is carry out vaccine trials, but this is a very long process. Another way to approach this question is to carry out cross-clade studies in the laboratory. In this study, cross-clade immune responses were investigated in thirty-nine HIV-1 subtype C-infected volunteers. An IFN-γ ELISpot assay was used using peptides based on vaccine candidates for subtype C (South African and Chinese), subtype B and subtypes A and D. A pool-matrix ELISpot approach was used in order to identify the individual reactive peptides. The peptides in pools and matrices that gave a positive HIV-specific response were tested individually in a confirmatory ELISpot assay for two highly reactive individuals.

Initially, validation of the assay was performed. There was a significant correlation between the magnitude of responses obtained in pools and those obtained in matrices. The inclusion of a QC sample helped in determining which assays passed, as well as in monitoring inter- and intra-assay variability.

The total magnitude of IFN-γ responses were not significantly different between peptides based on subtype C (South Africa and Chinese) peptides. These intra-clade responses were significantly higher than responses to other subtypes, namely subtypes B, A and D. These data suggest that even though subtype C-infected individuals mount robust IFN-γ responses against different HIV subtypes, there is preferential recognition of peptides based on the infecting subtype. Previous studies have also found the same results (McKinnon *et al.*, 2005; Yu *et al.*, 2005). Overall, there were substantial cross- and intra-clade HIV-specific T cell responses detected in this study. This is further supported by the high cross-clade reactivity ratios as well as highly significant Spearman correlation coefficients obtained for the Gag responses from the different subtypes.

Whether genetic distance between the infecting virus sequence and vaccine immunogen sequences has an impact on vaccine-induced immune response is important in vaccine design. In this study no correlation was obtained between the magnitude of HIV-specific IFN-γ responses and overall amino acid distances between the infecting Gag sequence and Gag peptide reagent sequences. This might be due to the fact that genetic distances encompassed other regions not important in eliciting immune responses, and that those regions are outside the epitopic region of the peptides.

An investigation of the distribution of the HIV-specific T cell immune responses across the Gag showed the p24 region contributes most of the responses. This is likely to be due to the fact that p24 is generally more conserved when compared to the p17 and p15 regions. This identification of the most immunogenic region of Gag is important in HIV vaccine immunogen selection since an ideal candidate vaccine will have to contain those regions of the HIV genome that can elicit strong T cell responses. However, there were some peptides from the p17 and p15 regions of Gag which gave rise to cross-clade responses of high magnitudes. These peptides may not be ignored in vaccine immunogen design since they make an important contribution to the total Gag-specific T cell response. This identification of Gag p24 as the most immunodominant region of HIV proteome has also been found by other researchers (Yu *et al.*, 2005).

A detailed evaluation of cross- and intra-clade cellular immune responses to HIV-1 Gag was performed for two of the volunteers. In these individuals, the five peptide variant sequences were compared to the infecting virus sequence. Some peptides were not recognized at all. Some peptide variants were not recognized may be due to variation within the epitopic regions that resulted in loss of recognition by the restricting HLA or loss of recognition by T cell receptors. In other cases, an individual recognized some variants of the peptides sets tested and not others. This might be due to the fact that some mutations can not be tolerated by HLA molecules if they are non-conservative and involve HLA anchor residues. In addition, the amino acid changes might be involving HIV-specific TCR contact sites, leading to lack of recognition. However, there were cases where there was recognition even though corresponding peptides differed in their amino acid sequences. This suggest that TCR of HIV-specific T cells can tolerate some degree of amino acid substitution, though analysis of these amino acid substitution showed that most of them were semi- or conservative substitutions (amino acids

being replaced by other amino acids of more or less the same charge, hydrophobicity and size). Another reason could be that the nature of amino acid substitutions could be tolerated by HLA anchor residues.

There were cases where the peptide sequences less closely related to the infecting sequence gave rise to a positive response while as the one closest or similar to the infecting sequence was not recognized. This might suggest escape from the cellular immune response. Possibly the recognized sequence was present before the day of sequencing which mutated due to immune pressure, and the peptide sequences were similar to this sequence before mutation hence recognition by HIV-specific T cells. Therefore, HIV-specific T cells could still recognize this sequence which they encountered first. Overall, these data suggest that variation outside the HLA anchor residues and TCR contact sites might have less impact on vaccine induced T cell reactivity. Furthermore, HLA anchor residues and TCR contact sites can tolerate some degree of amino acid substitution.

Results of the analysis of the breadth of responses from the individuals showed there was preferential recognition of peptides based on subtype C, as the individuals were infected with this subtype. The two individuals had broad HIV-specific T cell responses averaging 17 peptides. Most of these peptides came from Gag p24 region. The recognition of more p24 peptides than p17 or p15 might suggest that mutations within this region that result in abrogation of T cell responses might be those that the virus cannot tolerate due to fitness cost on replicative capacity. This results in less variation in this region of the virus. This is inline with results from a previous study which showed fitness cost in mutation in Gag p24 epitope TSTLQEQIW (Martinez-Picado *et al.*, 2006), which is found in peptide 58 recognized by individual CC22 with W replaced by A. The results on the breadth of the responses suggest that using different peptides sets in testing for HIV-specific T cell responses increases the breadth of detectable T cell responses as found in a previous study (Currier *et al.*, 2006). However, the breadth of the responses did not significantly differ among the different subtypes.

The most immunodominant peptide in the two individuals was peptide 72 (sequence KEPFRDYVDRFFKTL). This peptide has a high epitope density and has been identified previously as a highly immunodominant peptide to include in vaccines as the epitopes are

restricted by different HLA molecules, thereby covering people with different HLA backgrounds (Frahm *et al.*, 2007; Kiepiela *et al.*, 2007; Masemola *et al.*, 2004).

Further characterization of the individual reactive peptides in two individuals allowed the classification of the T cell responses detected in this study into five categories, namely those recognizing one, two, three, four, and five peptide sets. The significant difference between those T cell responses recognizing at least three peptide variants and those recognizing one or two (the former being higher than the latter) suggest that cross-clade HIV-specific T cell responses are focused towards peptides with low intra-clade entropy and simultaneously high inter-clade homology. The data suggest that among the five Gag peptide variants used in the study, corresponding viral regions share a similar degree of viral diversity. This is probably due to structural constraints that prevent sequence mutations in specific parts the *gag* gene.

In summary, cross-reactive HIV-specific T cells preferentially recognize peptides with low entropy and simultaneously high inter-clade homology. HIV-1 subtype C-infected individuals can mount substantial cross- and intra-clade immune responses. However, there was no difference in immune reactivity between South African or Chinese subtype C Gag peptides, suggesting that vaccines based on these immunogens would work equally well. On the other hand, the magnitude of responses to other subtypes was lower, suggesting that vaccines based on these subtypes may be less effective than subtype matched vaccines.

CHAPTER 4

4.1. DISCUSSION AND CONCLUSION

An effective HIV vaccine will need to protect against multiple HIV subtypes or recombinant forms. In the present study, full length *gag* sequences from forty HIV-infected volunteers were genetically characterized. Cellular immune responses in these individuals were assessed to determine their ability to recognise peptides from genetically diverse viral isolates representing multiple subtypes. The peptide reagents used in this study were based on five sequence variants representing four subtypes including South African subtype C ($C_{S.A}$, strain Du422), Chinese subtype C strain (C_{CH}), subtype B CAM-1 strain, and subtypes A and D. The subtype $C_{S.A}$ and A sequences used in this study are based on immunogens expressed by candidate HIV vaccines which are in the process of going to clinical trial or have been tested in humans (Burgers *et al.*, 2006; Hanke *et al.*, 2004).

The individuals in this study were all infected with subtype C viruses. This is in line with previous studies, that have shown that the major circulating viruses in South Africa are subtype C viruses (van Harmelen *et al.*, 1999). No recombination breakpoints were detected. This lack of recombination in these subtype C viruses should improve the prospects of vaccines based on subtype C *gag* sequences in that it is unlikely that they will fail due to the emergence of escape recombinants expressing vaccine targeted epitopes derived from non-subtype C viruses (Bredell *et al.*, 2007). As shown with previous studies, there is clear evidence of geographical clustering of subtype C isolates from various parts of the world (Bredell *et al.*, 2007), with Brazilian, Indian and Chinese sequences forming well-supported monophyletic groups in the *gag* subtype C phylogenetic tree. This indicated that these subtype C epidemic in different regions of the world are genetically distinct. Studies on subtype C sequences from China have shown that these viruses are BC recombinant viruses; however, their *gag* gene is derived from subtype C.

Studies have shown that individuals can mount cross-reactive T cell responses (Cao *et al.*, 2000; Ferrari *et al.*, 1997; Buseyne *et al.*, 1998). However, many of these earlier cross-clade studies have focused on a limited number of selected epitopes. Moreover, the studies relied on the use of pools of overlapping peptides or cells infected with vaccinia virus constructs expressing the entire HIV proteins and therefore could not assess the degree of cross-clade recognition at the peptide or epitope level (Cao *et al.*, 2000; Ferrari *et al.*, 1997; Buseyne *et al.*,

1998, Currier *et al.*, 2003). More recent studies have looked at cross-clade immune responses at the peptide level (Yu *et al.*, 2005). However, sequence data was not generated in most of these recent studies to allow additional characterization of these cross-clade HIV-specific T cell responses.

We have investigated T cell reactivity of 39 individuals whereby the sequence of the infecting virus is known. In addition, we have assessed responses against Gag peptides from five sequence variants, South African C, Chinese C, subtypes B, A and D at the single peptide level. Although South Africa and China have genetically distinct subtype C epidemics, in this study, the magnitude of HIV-specific T cell responses to Chinese and South African subtype C peptide variants did not significantly differ. However, the magnitude of the responses to these two subtypes C peptide variants was significantly higher than that of Gag peptide reagents based on subtypes B, A and D sequences. This is further corroboration of results from previous studies which have shown that HIV-specific T cells are cross-reactive among different HIV subtypes but with preference to the circulating subtype (McKinnon *et al.*, 2005; Geldmacher *et al.*, 2007). The magnitude of response to the five peptide variants were in the order C_{CH}> $C_{S.A}$>B>A>D. These data suggest that vaccines based on these HIV-1 subtype C sequences (subtype-matched vaccines) may work equally well in HIV-1 subtype C-infected individuals from different regions in the world. In addition, although vaccines based on other HIV subtypes may still induce cross-reactive responses, this reactivity may be less in subtype-mismatched vaccines.

Further dissection of the $CD8^+$ T-cell immune responses to the three individual Gag proteins showed the major contribution of Gag protein 24 (p24). This is likely to be due to the conserved nature of this region of the Gag protein. Interestingly, p17 and p15 had an important contribution to the responses and had regions that mounted cross-clade immune responses despite them being variable. It will be interesting to see whether these cross-clade responses relate to vaccine induced cross-clade immunity.

The results from this study demonstrate that within a single study individual, some HIV peptides can be exclusively recognized in the clade C sequence variants (South African C and Chinese C) while others were uniquely recognized in the clade A, B and D peptide variants. This likely reflects the overall sequence diversity within subtype C. However, the recognition

of clade A, B and D peptide variants and not the corresponding clade C peptide variants is of importance in the study of HIV-1-specific T cell immune responses using the ELISpot assay. This reflects that the use of different peptide variants increases the number of responses that can be detected compared to if only one peptide variant had been used.

An important analysis for vaccine design is the relationship between the genetic distance of the infecting sequence and the peptide reagent sequences that were used in the ELISpot assay and the magnitude of IFN-γ response. Although there was a weak negative association between the magnitude of response and amino acid distances, there was no significant correlation between the two. However, HIV-specific T cells recognise epitopes, only very short protein regions. Amino acid distances used were for the full length Gag peptide sequences and included sequences outside of the epitopes. Variation in these less important regions might have affected the results. Therefore it was necessary to analyse individual peptide variants.

Further characterization of peptides used in the study identified viral regions with low intra-clade diversity and simultaneously high inter-clade homology that were preferentially recognized by T-cells among all the five peptide variant sequences. This analysis allowed the classification of all the individual reactive peptides in two individuals into five categories that is those that were recognized in one, two, three, four and five peptide variants. Indeed, those peptides that were recognized in three or more peptide variants had significantly lower entropy scores and simultaneously high percentage homology when compared to peptides recognized in one or two peptide variants. Overall, these data suggest that within the clade A, B, C and D sequences, some corresponding viral regions share a similar degree of viral diversity, possibly due to structural constraints that prevent sequence mutations in specific parts of the viral genome. Some of these cross-recognized peptides were identical across the five peptide variants and their cross-recognition was therefore evident. Yet, cross-clade recognition of peptides with considerable differences in their amino acid composition was also observed, where most of the amino acid changes were conserved or semi-conserved. In other words, an amino acid was replaced with one that falls in the same charge or hydrophobic category. This suggests that T cell receptors of HIV-specific T-cells as well as HLA molecules can tolerate some degree of amino acid substitution in their epitopes without total loss of epitope recognition or binding as previously found in other studies (McKinney *et al.*, 2004; Addo *et al.*, 2003).

The study presented in this thesis was limited in that only two individuals were investigated for the single reactive peptides responsible for the Gag responses. This was due to time constraints. Further work on the study will complete the identification of peptides predicted from the screening ELISpot assay in the remaining 37 study individuals. This will allow for more depth analysis of breadth of responses to the different subtypes, as well as comparisons with the infecting viral subtype.

This study only investigated the monofunctional aspect of the cellular immune response, namely the production of IFN-γ. It will be interesting to determine the production of other cytokines such as TNF-α and functions such as cytotoxicity to see whether they have higher cross-reactivity than IFN-γ production association with better clinical outcome. This is because the immune correlates of viral control are not yet clear, but recent studies suggest the importance of polyfunctional $CD8^+$ T cells (Betts *et al.*, 2006) and therefore, will be important to know whether polyfunctional HIV-specific $CD8^+$ T cell are associated with higher cross-reactivity.

In conclusion, this study showed that subtype C infected individuals recognized peptides based on Chinese and South African sequences equally suggesting that it will not be necessary to design vaccines based on regional variation. In addition, while extensive cross-clade recognition was detected, the total magnitude of cross-reactive T cell responses was lower than that of intra-clade T cell responses suggesting that there are some advantages of matching a vaccine to circulating subtypes. However, the inclusion of new generation of vaccines that include immunogens such as T cell mosaic antigens that reduce the effect of diversity would potentially be beneficial to enhancing effective vaccine-induced cross-clade responses (Fischer *et al.*, 2007).

APPENDICES

Appendix A: Molecular Biology Techniques

A1: RNA Extraction

- Samples were equilibrated at room temperature (15-20^{0}C).
- Buffer AVE was also equilibrated at room temperature for elution in step 10.
- The precipitate in buffer AVL/Carrier RNA by heating, if necessary, and cool to room temperature before use.
- All centrifugation steps were carried out at room temperature.

1. 560µl of prepared buffer AVL containing Carrier RNA was pipetted into a 1.5-ml micro centrifuge tube.
2. 140µl of plasma was added, mixed by pulse vortexing for 15 seconds.
3. The mixture incubated at room temperature (15-25^{0}C) for 10 minutes.
4. This was brief centrifuged to remove drops from the inside of the lid.
5. 560µl of 96-100 % ethanol was added to the tubes, pulse-vortexed for 15 seconds and brief centrifuged.
6. 630µl of the resulting solution was applied to QIAamp spin column in a 2ml collection tube without wetting the rim and centrifuged at 6000 × *g*; 8000 rpm for 1 minute. The spin column was placed into a clean 2ml collection tube and tube containing the filtrate was discarded.
7. The QIAamp spin column was carefully opened and step 6 repeated.
8. 500µl of buffer AW1 was added and centrifugation performed at 6000 × *g*; 8000 rpm for 1 minute. The column was placed into another clean 2ml collection tube and the filtrate was discarded.
9. 500µl of buffer AW2 was added and centrifuged at full speed 20 000×*g;* 14 000 rpm for 3 minutes.

9a Spin column was placed into a clean 2ml collection tube and centrifuged at full speed for 1 minute.

10. The QIAamp spin column was placed into a clean 1.5ml micro centrifuge tube and 60µl of elution buffer AVE was added, incubated for 1 minute at room temperature and then centrifuged at 6000 × *g*; 8000 rpm for 1 minute.

A2: PCR purification of amplicons

A protocol designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions. Fragments ranging from 100bp-10kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a micro centrifuge.

- Ethanol (96-100%) was added to buffer PE before use.
- All centrifuge steps were carried at 13 000 rpm (~17 900 *x g*) in a conventional tabletop micro centrifuge.

1 5 volumes (200µl) of buffer PB was added to 1 volume (40µl) of the PCR sample mix.
2 A QIAquick spin column was placed in a provided 2 ml collection tube
3 The sample was applied to the QIAquick column to bind DNA and spun for 60 seconds.
4 The flow through was discarded and the QIAquick column placed into the same tube.
5 0.75 ml of buffer PE was added to the QIAquick column and centrifuged for 60 seconds to wash.
6 The flow through was discarded and the column placed back into the same tube. This was centrifuged for an additional 1 minute.
7 The QIAquick column was placed into a clean 1.5 ml micro centrifuge tube.
8 50µl of buffer EB (10mM Tris-Cl, pH 8.5) was added to the centre of the QIAquick membrane and centrifuged for I minute to elute DNA.

The eluted DNA was stored at -20^{0}C.

A4: Roche Molecular Weight marker VI (Roche, GmbH, Mannheim, Germany)

For example, sample band intensity corresponding to fragment 453 (11ng) in the Molecular weight marker lane, 5µl of sample was loaded therefore the concentration of sample would be:

$0.75 \times 11ng \div 5\mu l$

$=1.65ng/\mu l$

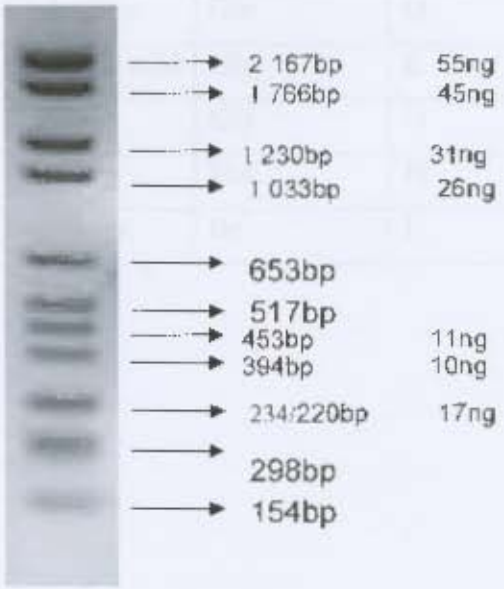


Figure A1 Molecular weight marker VI (Roche, GmbH: Mannheim, Germany). A known quantity of the marker is run in parallel with study sample amplicon to allow for quantification by band intensity comparison.

Table A1 Amino acid codes

Amino acid	3-letter code	IUB code	Amino acid	3-letter code	IUB code
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenyalanine	Phe	F
Cystine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

Table A2 Codons

3-letter code	Unambiguous codons	IUB codons
Ala	GCT, GCC, GCA, GCG	GCX
Asp, Asn	GAT, GAC, AAT, AAC	RAY
Cys	TGT, TGC	TGY
Asp	GAT, GAC	GAY
Glu	GAA, GAG	GAR
Phe	TTT, TTC	TTY
Gly	GGT, GGC, GGA, GGG	GGX
His	CAT, CAC	CAY
Ile	ATT, ATC, ATA	ATH
Lys	AAA, AAG	AAR
Leu	TTG, TTA, CTT, CTC, CTA, CTG	TTR CTX
Met	ATG	ATG
Asn	AAT, AAC	AAY
Pro	CCT, CCC, CCA, CCG	CCX
Gln	CAA, CAG	CAR
Arg	CGT, CGC, CGA, CGG, AGA, AGG	CGX AGR
Ser	TCT, TCC, TCA, TCG, AGT, AGC	TCX AGY
Thr	ACT, ACC, ACA, ACG	ACX
Val	GTT, GTC, GTA, GTG	GTX
Trp	TGG	TGG
XXX		XXX
Tyr	TAT, TAC	TAY
Glu, Gln	GAA, GAG, CAA, CAG	SAR
End	TAA, TAG TGA	TAR TRA

Appendix B: Molecular Biology Reagents and recipes

B1: 10X TBE Buffer

108g Tris-HCl

55g Boric acid

20ml 1.5M EDTA

made up to 1 litre with dH_2O

B2: 6X Agarose Gel Eletrophoresis Loading Dye

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol

In deionized water

B3: Sequencing buffer

200mM Tris, pH 9.0

5mM $MgCl_2$

B4: Recombination analysis

Each of the study participants' full length *gag* sequence was analyzed for recombination using REGA version 6.4.1, a software program which compares each nucleotide position to other HIV-1 pure subtype and CRF reference sequences to determine whether there is sufficient phylogenetic signal to classify the sequence as a pure subtype of CRF, recombinant viruses or unclassified viral subtypes (de Oliveira *et al.*, 2005).

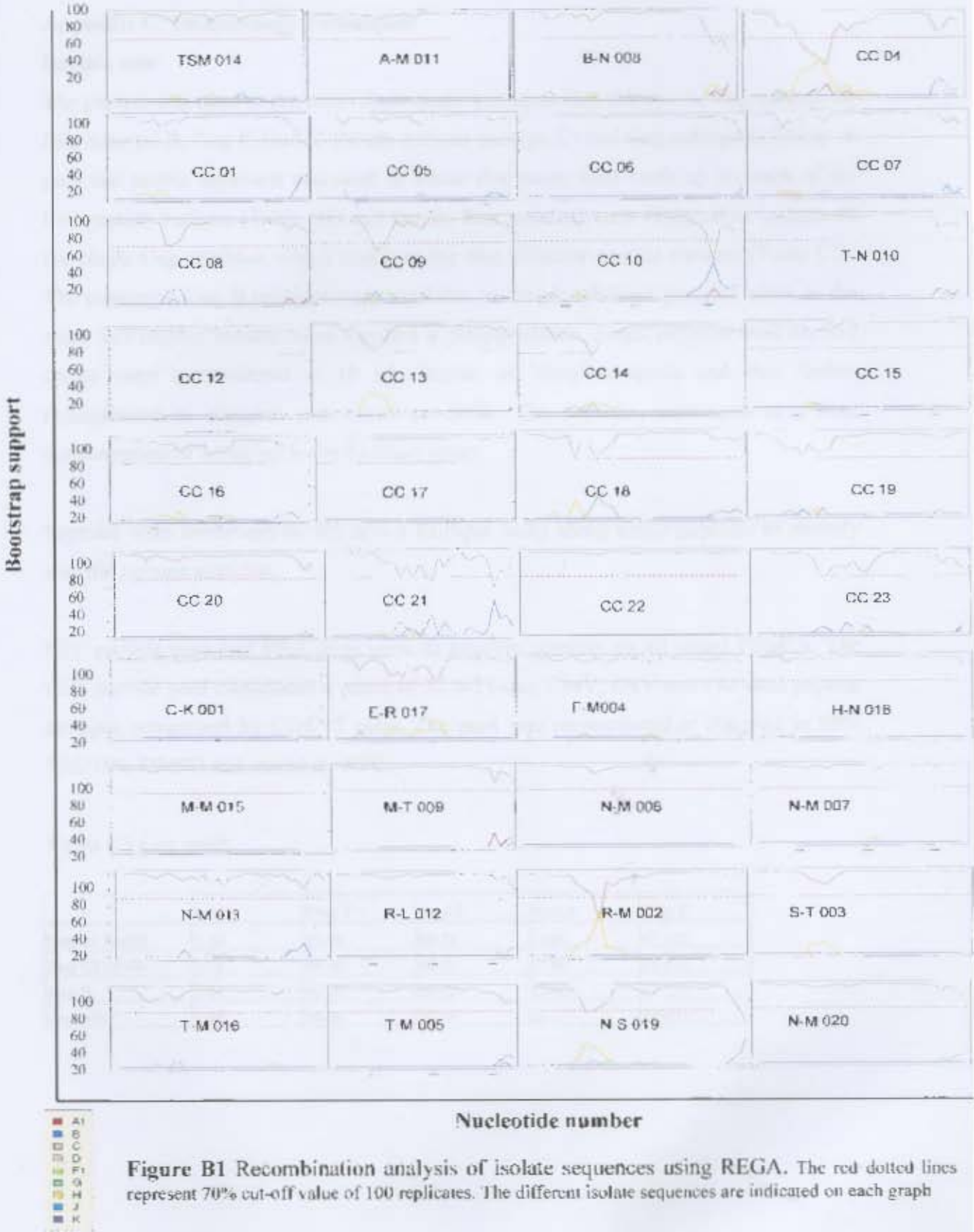


Figure B1 Recombination analysis of isolate sequences using REGA. The red dotted lines represent 70% cut-off value of 100 replicates. The different isolate sequences are indicated on each graph

Appendix C: Immunology Techniques

Peptide sets

The peptide sets used in the cross-clade study belong to Gag subtype A, Gag subtype D, Gag subtype B, Gag C Du422 (South African subtype C) and Gag subtype C China. A pool and matrix approach was used in which five pools were made up for each of the five peptide variants (Table C1) and twenty-four matrices were designed to include all the single Gag peptides, which make up the five different peptide variants (Table C2). The consensus Gag B peptides were available as 1mg lyophilized peptides while as the other four peptide variants were supplied at 500µg/peptide. Single peptides from the five clades were reconstituted to 10 µl aliquots of 10mg/ml stocks and then further reconstituted to 30µg/ml and stored at -80^{0}C. The peptides were used at a final concentration of 1.5µg/ml in the ELISpot assay.

Peptides were confirmed by the IFN-γ ELISpot assay using single peptides to identify specific epitope stretches.

CEF peptide pool and PHA were used as positive controls on all tested PBMCs. The CEF peptide pool constituted a panel of 32 8-11-mer CMV, EBV and Flu virus peptide epitopes recognized by CD8$^+$ T cells. The pool was reconstituted at 20µg/ml in 90% PBS/10% DMSO and stored at -80^{0}C.

Table C1 Gag pools

	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
Gag C1 Du422	1-24	25-48	49-72	73-96	97-120
Gag C2 China	1-24	25-48	49-72	73-96	97-119
Gag B	1-24	25-48	49-72	73-96	97-123
Gag A/D	1-18	19-36	37-54	55-72	73-90

Table C2 Gag matrices

M1	M2	M3	M4	M5	M6	M7	M8
Gag C1-1	Gag C1-2	Gag C1-3	Gag C1-4	Gag C1-5	Gag C1-6	Gag C1-7	Gag C1-8
Gag C1-25	Gag C1-26	Gag C1-27	Gag C1-28	Gag C1-29	Gag C1-30	Gag C1-31	Gag C1-32
Gag C1-49	Gag C1-50	Gag C1-51	Gag C1-52	Gag C1-53	Gag C1-54	Gag C1-55	Gag C1-56
Gag C1-73	Gag C1-74	Gag C1-75	Gag C1-76	Gag C1-77	Gag C1-78	Gag C1-79	Gag C1-80
Gag C1-97	Gag C1-98	Gag C1-99	Gag C1-100	Gag C1-101	Gag C1-102	Gag C1-103	Gag C1-104
Gag C2-1	Gag C2-2	Gag C2-3	Gag C2-4	Gag C2/C1-5	Gag C2/C1-6	Gag C2-7	Gag C2-8
Gag C2-25	Gag C2-26	Gag C2-27	Gag C2-28	Gag C2-29	Gag C2-30	Gag C2-31	Gag C2/C1-32
Gag C2/C1-49	Gag C2-50	Gay C2-51	Gag C2-52	Gag C2-53	Gag C2/C1-54	Gag C2/C1-55	Gag C2/C1-56
Gag C2/C1-73	Gag C2-74	Gag C2-75	Gag C2-76	Gag C2-77	Gag C2/C1-78	Gag C2/C1-79	Gag C2/C1-80
Gag C2/C1-97	Gag C2/C1-98	Gag C2/C1-99	Gag C2/C1-100/B-102	Gag C2/C1-101/B-103	Gag C2/C1-102/B-104	Gag C2/C1-103/B105	Gag C2/C1-104/B106
Gag B-2	Gag B-3	Gag B-4	Gag B-5	Gag B-6	Gag B-7	Gag B-8	Gag B-9
Gag B-26	Gag B-27	Gag B-28	Gag B-29	Gag B-30	Gag B-31	Gag B-32	Gag B-33
Gag B-50	Gag B-51	Gag B-52	Gag B-53	Gag B-54	Gag B-55	Gag B-56	Gag B-57
Gag B-74	Gag B-75	Gag B-76	Gag B-77	Gag B-78	Gag B-79	Gag B-80	Gag B-81
Gag B-99	Gag B-100	Gag B-101	Gag B-102/C1/C2-100	Gag B-103/C1/C2-101	Gag B-104/C1/C2-102	Gag B-105/C1/C2-103	Gag B-106/C1/C2-104
Gag B123	Gag B 95						
Gag A-17/C1/C2-49	Gag A-18/C1-50	Gag A-19/C1-51	Gag A-20	Gag A-21	Gag A-22	Gag A-23	Gag A-24/C1/C2-56
Gag A-41/C1/C2-73	Gag A-42/C1-74	Gag A-43/C1-75	Gag A-44	Gag A-45	Gag A-46	Gag A-47	Gag A-48
Gag A-65	Gag A-66	Gag A-67	Gag A-68	Gag A-69	Gag A-70	Gag A-71	Gag A-72
Gag A-89	Gag A-90						
Gag D-17	Gag D-18	Gag D/A-19/C1-51	Gag D-20	Gag D-21	Gag D-22	Gag D-23	Gag D/A-24/C1/C2-56
Gag D-41	Gag D-42	Gag D-43	Gag D-44	Gag D-45	Gag D/A-46	Gag D-47	Gag D-48
Gag D/A-65	Gag D/A-66	Gag D/A-67					
Gag D-83	Gag D-84	Gag D-85	Gag D 86	Gag D-87	Gag D 88	Gag D 89	Gag D-90
18	20	17	19	18	15	17	14

Table C2 *continued*

	M10	M11	M12	M13	M14	M15	M16
Gag C1-9	Gag C1-10	Gag C1-11	Gag C1-12	Gag C1-13	Gag C1-14	Gag C1-15	Gag C1-16
Gag C1-33	Gag C1-34	Gag C1-35	Gag C1-36	Gag C1-37	Gag C1-38	Gag C1-39	Gag C1-40
Gag C1-57	Gag C1-58	Gag C1-59	Gag C1-60	Gag C1-61	Gag C1-62	Gag C1-63	Gag C1-64
Gag C1-81	Gag C1-82	Gag C1-83	Gag C1-84	Gag C1-85	Gag C1-86	Gag C1-87	Gag C1-88
Gag C1-105	Gag C1-106	Gag C1-107	Gag C1-108	Gag C1-109	Gag C1-110	Gag C1-111	Gag C1-112
Gag C2-9	Gag C2/C1-10	Gag C2/C1-11	Gag C2/C1-12	Gag C2-13	Gag C2-14	Gag C2-15	Gag C2-16
Gag C2/C1-33/B-34	Gag C2/C1-34	Gag C2/C1-35	Gag C2/C1-36	Gag C2/C1-37	Gag C2/C1-38	Gag C2/C1-39	Gag C2/C1-40
Gag C2/C1-57	Gag C2-58	Gag C2-59	Gag C2-60	Gag C2-61	Gag C2-62	Gag C2-63	Gag C2-64
Gag C2/C1-81	Gag C2-82	Gag C2-83	Gag C2-84	Gag C2-85	Gag C2-86	Gag C2-87	Gag C2-88
Gag C2/C1-105/B107	Gag C2/C1-106/B108	Gag C2/C1-107/B109	Gag C2-108	Gag C2-109	Gag C2-110	Gag C2-111	Gag C2/C1-112
Gag B-10	Gag B-11	Gag B-12	Gag B-13	Gag B-14	Gag B-15	Gag B-16	Gag B-17
Gag B-34/C1/C2-33	Gag B-35	Gag B-36	Gag B-37	Gag B-38	Gag B-39	Gag B-40	Gag B-41
Gag B-58	Gag B-59	Gag B-60	Gag B-61	Gag B-62	Gag B-63	Gag B-64	Gag B-65
Gag B-82	Gag B-83	Gag B-84	Gag B-85	Gag B-86	Gag B-87	Gag B-88	Gag B-89
Gag B-107/C1/C2-105	Gag B-108/C1/C2-106	Gag B-109/C1/C2-107	Gag B-110/C2-108	Gag B-111/C2-109	Gag B-112/C2-110	Gag B-113	Gag B-114
Gag A-1	Gag A-2	Gag A-3	Gag A-4	Gag A-5/C1/C2-37	Gag A-6/C1/C2-38	Gag A-7/C1/C2-39	Gag A-8
Gag A-25/C1/C2-57	Gag A-26	Gag A-27	Gag A-28	Gag A-29	Gag A-30/C1-62	Gag A-31/C1-63	Gag A-32/C1-64
Gag A-49	Gag A-50	Gag A-51/C1-83	Gag A-52/C1-84	Gag A-53/C1-85	Gag A-54/C1-86	Gag A-55	Gag A-56
Gag A-73	Gag A-74	Gag A-75	Gag A-76	Gag A-77	Gag A-78	Gag A-79	Gag A-80
Gag D-1/C1/C2-33	Gag D-2/C1/C2-34	Gag D-3/C1/C2-35	Gag D-4/C1/C2-36	Gag D/A-5/C1/C2-37	Gag D/A-6/C1/C2-38	Gag D/A-7/C1/C2-39	Gag D/A-8
Gag D/A-25/C1/C2-57	Gag D-26/C1-58	Gag D-27/C1-59	Gag D-28/C1-60	Gag D-29	Gag D-30	Gag D-31	Gag D-32/C2-64
Gag D-49	Gag D-50	Gag D-51	Gag D-52	Gag D/A-53/C1-85	Gag D-54/C2-86	Gag D-55/C2-87	Gag D-56/C2-88
	Gag D-65/C1-10	Gag D-69/C1-11	Gag D-70/C1-12	Gag D-71	Gag D-72	Gag D-73	Gag D-74
Ψ 13	16	15	16	17	16	18	17

Table C2 *continued*

M17	M18	M19	M20	M21	M22	M23	M24
Gag C1-17	Gag C1-18	Gag C1-19	Gag C1-20	Gag C1-21	Gag C1-22	Gag C1-23	Gag C1-24
Gag C1-41	Gag C1-42	Gag C1-43	Gag C1-44	Gag C1-45	Gag C1-46	Gag C1-47	Gag C1-48
Gag C1-65	Gag C1-66	Gag C1-67	Gag C1-68	Gag C1-69	Gag C1-70	Gag C1-71	Gag C1-72
Gag C1-89	Gag C1-90	Gag C1-91	Gag C1-92	Gag C1-93	Gag C1-94	Gag C1-95	Gag C1-96
Gag C1-113	Gag C1-114	Gag C1-115	Gag C1-116	Gag C1-117	Gag C1-118	Gag C1-119	Gag C1-120
Gag C2-17	Gag C2-18	Gag C2-19	Gag C2 20	Gag C2-21	Gag C2-22	Gag C2 23	Gag C2-24
Gag C2/C1-41	Gag C2/C1-42	Gag C2/C1-43	Gag C2/C1-44	Gag C2/C1-45	Gag C2/C1-46	Gag C2/C1-47	Gag C2/C1-48
Gag C2/C1-65	Gag C2/C1-66	Gag C2/C1-67	Gag C2-68	Gag C2-69	Gag C2-70	Gag C2-71	Gag C2/C1-72
Gag C2-89	Gag C2 90	Gag C2-91	Gag C2-92	Gag C2-93	Gag C2-94	Gag C2-95	Gag C2/C1-96
Gag C2/C1-113	Gag C2/C1-114	Gag C2 115	Gag C2-116	Gag C2-117	Gag C2-118	Gag C2-119	Gag B-1
Gag B-18	Gag B 19	Gag B-20	Gag B-21	Gag B-22	Gag B-23	Gag B-24	Gag B-25
Gag B-42	Gag B-43	Gag B-44	Gag B-45	Gag B-46	Gag B-47	Gag B-48	Gag B 49
Gag B-66	Gag B-67	Gag B-68	Gag B-69	Gag B-70	Gag B-71	Gag B-72	Gag B-73
Gag B-90	Gag B-91	Gag B-92	Gag B-93	Gag B-94	Gag B-96	Gag B-97	Gag B-98
Gag B-115	Gag B-116	Gag B-117	Gag B-118	Gag B 119	Gag B-120	Gag B-121	Gag B-122
Gag A 9	Gag A-10	Gag A-11	Gag A 12	Gag A 13	Gag A 14	Gag A-15	Gag A-16/C1/C2-48
Gag A-33/C1/C2-65	Gag A-34/C1/C2-66	Gag A-35/C1/C2-67	Gag A-36/C1-68	Gag A-37/C1-69	Gag A-38/C1-70	Gag A-39/C1-71	Gag A-40/C1/C2-72
Gag A-57	Gag A-58	Gag A-59	Gag A-60	Gag A-61	Gag A-62	Gag A-63	Gag A-64
Gag A 81	Gag A-82	Gag A 83	Gag A-84	Gag A-85	Gag A-86	Gag A-87	Gag A-88
Gag D/A-9	Gag D/A-10	Gag D-11/C1/C2-43	Gag D-12/C1/C2-44	Gag D-13/C1/C2-45	Gag D-14/C1/C2-46	Gag D-15	Gag D-16
Gag D/A-33/C1/C2-65	Gag D/A-34/C1/C2-66	Gag D/A-35/C1/C2-67	Gag D/A-36/C1-68	Gag D/A-37/C1-69	Gag D/A-38/C1-70	Gag D/A-39/C1-71	Gag D-40
Gag D 57	Gag D-58	Gag D/A-59	Gag D/A-60	Gag D/A-61	Gag D/A-62	Gag D/A-63	Gag D/A-64
Gag D-75	Gag D-76	Gag D-77	Gag D-78	Gag D-79	Gag D 80	Gag D-81	Gag D-82
Ψ 17	17	17	18	18	18	19	17

Common peptides between C_1 (Du422) and C_2 (Chinese)
No peptide

Ψ Number in the last raw of each table indicate the number of different peptides

Appendices

Specimens

A total of 40 asymptomatic HIV-1 subtype C infected individuals were enrolled in the study: 20 samples were stored at NICD sample repository and the other 20 at the IIDMM

Assay protocol

The ELISpot assays were conducted for screening of peptide responses using a panel of peptide described in C1 and C2. PBMC from NICD blood donor (QC sample-NICD 063) was used as a positive control sample for each plate. The QC sample had been tested against PHA and CEF and was a known responder and therefore used as a positive control.

Participants' PBMCs were thawed as described in Chapter 3 and tested in duplicate against each Gag peptide pool, once against each Gag matrix and twice against CEF and PHA (plate layout in Table C3). Eight negative control wells, four positive control wells and four peptide control wells were used.

The negative control wells consisted of six unstimulated PBMC and two unstimulated wells for the QC sample per plate. Each plate also had six wells containing R10 (media only).

Positive control wells consisted of two PHA stimulated PBMC and two PHA stimulated wells for the QC sample per plate. The control wells consisted of two CEF stimulated PBMC and two CEF stimulated QC sample on each plate.

Peptide confirmations were performed for those peptides that gave a positive response after the screening ELISpot assays.

Table C3 ELISpot worksheet and plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
a	Gag C1 Pool 1	Gag C1 Pool 1	Gag C2 Pool 1	Gag C2 Pool 1	Gag B Pool 1	Gag B Pool 1	Gag A Pool 1	Gag A Pool 1	Gag D Pool 1	Gag D Pool 1	Cells - Media	Cells + Media
b	Gag C1 Pool 2	Gag C1 Pool 2	Gag C2 Pool 2	Gag C2 Pool 2	Gag B Pool 2	Gag B Pool 2	Gag A Pool 2	Gag A Pool 2	Gag D Pool 2	Gag D Pool 2	Cells - Media	Cells + Media
c	Gag C1 Pool 3	Gag C1 Pool 3	Gag C2 Pool 3	Gag C2 Pool 3	Gag B Pool 3	Gag B Pool 3	Gag A Pool 3	Gag A Pool 3	Gag D Pool 3	Gag D Pool 3	Cells - Media	Cells + Media
d	Gag C1 Pool 4	Gag C1 Pool 4	Gag C2 Pool 4	Gag C2 Pool 4	Gag B Pool 4	Gag B Pool 4	Gag A Pool 4	Gag A Pool 4	Gag D Pool 4	Gag D Pool 4	Media	Media
e	Gag C1 Pool 5	Gag C1 Pool 5	Gag C2 Pool 5	Gag C2 Pool 5	Gag B Pool 5	Gag B Pool 5	Gag A Pool 5	Gag A Pool 5	Gag D Pool 5	Gag D Pool 5	Media	Media
f	Gag M1	Gag M2	Gag M3	Gag M4	Gag M5	Gag M6	Gag M7	Gag M8	Gag M9	Gag M10	Gag M11	Gag M12
g	Gag M13	Gag M14	Gag M15	Gag M16	Gag M17	Gag M18	Gag M19	Gag M20	Gag M21	Gag M22	Gag M23	Gag M24
h	CEF	CEF	PHA	PHA	Media	Media	QC Cells - Media	QC Cells + Media	QC CEF	QC CEF	QC PHA	QC PHA

			Reagent number	Initials		Start Time	End Time	Initials
Ptid		Coating Ab			Blocking	:	:	
CTL No		Secondary Ab			O/N incubation	:	:	
Visit		Streptavidin			2nd Ab	:	:	
Cells/Well		Nova Red			Streptavidin	:	:	
QC ID		Blocking media			Nova Red	:	:	
Kit Lot No		FBS			Plate Number			
Ref: RLT0014		Coating Date			Plate Read By		Date	

Test acceptance criteria

A test was regarded as positive when the response was >100 sfu/106 PBMCs and at least 3 times the mean background response. The positive response in the pool was supposed to match a response in a matrix pool that shares one of the peptides in the pool.

Fail criteria for the ELISpot assay included any one or more of the following:

Greater than 100 spots in the negative control wells

Grater than 5 spots per well for the wells containing media only

Less than 400 spots per well for the PHA wells.

Record keeping

An ELISpot worksheet (Table C3) was completed with each assay performed. The plates were read by the CTL Immunospot Analyzer and data saved on CD plates. All completed worksheets and ELISpot raw data and calculated data were archived at the UCT human Immunology Laboratory and copies sent to NICD Immunology Laboratory.

Table C3 ELISpot worksheet and plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
a	Gag C1 Pool 1	Gag C1 Pool 1	Gag C2 Pool 1	Gag C2 Pool 1	Gag B Pool 1	Gag B Pool 1	Gag A Pool 1	Gag A Pool 1	Gag D Pool 1	Gag D Pool 1	Cells + Media	Cells + Media
b	Gag C1 Pool 2	Gag C1 Pool 2	Gag C2 Pool 2	Gag C2 Pool 2	Gag B Pool 2	Gag B Pool 2	Gag A Pool 2	Gag A Pool 2	Gag D Pool 2	Gag D Pool 2	Cells + Media	Cells + Media
c	Gag C1 Pool 3	Gag C1 Pool 3	Gag C2 Pool 3	Gag C2 Pool 3	Gag B Pool 3	Gag B Pool 3	Gag A Pool 3	Gag A Pool 3	Gag D Pool 3	Gag D Pool 3	Cells + Media	Cells + Media
d	Gag C1 Pool 4	Gag C1 Pool 4	Gag C2 Pool 4	Gag C2 Pool 4	Gag B Pool 4	Gag B Pool 4	Gag A Pool 4	Gag A Pool 4	Gag D Pool 4	Gag D Pool 4	Media	Media
e	Gag C1 Pool 5	Gag C1 Pool 5	Gag C2 Pool 5	Gag C2 Pool 5	Gag B Pool 5	Gag B Pool 5	Gag A Pool 5	Gag A Pool 5	Gag D Pool 5	Gag D Pool 5	Media	Media
f	Gag M1	Gag M2	Gag M3	Gag M4	Gag M5	Gag M6	Gag M7	Gag M8	Gag M9	Gag M10	Gag M11	Gag M12
g	Gag M13	Gag M14	Gag M15	Gag M16	Gag M17	Gag M18	Gag M19	Gag M20	Gag M21	Gag M22	Gag M23	Gag M24
h	CEF	CEF	PHA	PHA	Media	Media	QC Cells + Media	QC Cells + Media	QC CEF	QC CEF	QC PHA	QC PHA

Ptid _____
CTL No _____
Visit _____
Cells/Well _____
QC ID _____
Kit Lot No _____
Ref: RLT0014

	Reagent number	Initials
Coating Ab		
Secondary Ab		
Streptavidin		
Nova Red		
Blocking media		
FBS		
Coating Date		

	Start Time	End Time	Initials
Blocking	:	:	
O/N incubation	:		
2nd Ab	:	:	
Streptavidin	:	:	
Nova Red		:	
Plate Number			
Plate Read By		Date	

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